

# **The Prognostic and Therapeutic Significance of Biological Molecular Markers in Human Anorectal Carcinomas treated with 5'Fluorouracil Chemoradiation**

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## **Abstract**

Advances in tumour biology have led to studies of new biological markers, which may predict treatment response and clinical outcome independently of standard clinicopathological variables. An immunohistochemical analysis of a panel of molecular markers was studied, in a series of anorectal tumours, treated with 5'fluorouracil (5FU) chemoradiation.

240 tumour samples from the ACT I anal cancer trial were analysed. Patients were randomised to radiation alone (RT) or concurrent mitomycin/5FU/RT (CMT). On multivariate analyses tumour stage, treatment response, CD34 (vascularity), thymidine phosphorylase (TP) and p53 expression were independent predictors of clinical outcome. Tumour stage and p53 expression were associated with a poorer response to treatment in both randomisation arms. Increasing cyclin A and decreasing bcl-2 predicted for an improved response to radiation alone whilst CD34, tumour stage and TP expression predicted for an improved survival in the CMT arm.

Archived tumour samples from 60 patients with locally advanced rectal carcinoma were also studied. On multivariate analyses resection margin, nodal stage, treatment response, EGFR and p53 expression were independent predictors of clinical outcome. Increasing TP and cyclin A expression were associated with an improved response to chemoradiation.

The response of TP and thymidylate synthase (TS) to radiation was investigated in two colon carcinoma cell lines. TP activity significantly increased in response to a single and fractionated RT dose and TS significantly decreased. This may explain the lack of prognostic significance of TS and confirms that capecitabine, which is activated by TP in tumour cells, is a rational agent to combine with radiation.

In conclusion survival and treatment response in anorectal carcinomas can be predicted independently of standard clinicopathological characteristics. They can also predict survival between different therapeutic modalities, which may lead to improved chemoradiation strategies. Tailoring the treatment to the individual may become a future possibility.

## **Awards and Prizes from the work of this Thesis**

**2004 RCR Ross Award:** Awarded at the 2004 Annual British Cancer Research Meeting, Manchester, for best oral presentation. 'The role of biological molecular markers in predicting response to treatment and outcome in squamous cell carcinoma of the anus'.

**2004 ASCO Merit Award:** awarded by competitive application of an original research paper ('The role of biological molecular markers in predicting both response to treatment and clinical outcome in squamous cell carcinoma of the anus') to the American Society of Clinical Oncology, Annual Scientific GI Symposia Meeting in San Francisco, USA, January 2004.

**2004 Sanofi Oncology Award:** awarded by competitive application to permit presentation of proffered paper at the American Society of Clinical Oncology Meeting 2004.

**2002 Royal College of Radiologists Pump Prime Grant Award:** awarded by competitive application of a research grant submission: 'Defining Predictive Molecular Markers for the effectiveness of Chemoradiation in Anal Carcinoma'.



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**Mawdsley, S.K.** Molecular markers in rectal cancer: Can we predict response to treatment and outcome? – Royal Society of Medicine Current Medical Literature Series, *Gastroenterology*, 2004, Vol. 23, 2:25-31.

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## Abbreviations

AJCC	American Joint Committee on Cancer
CA9	Carbonic Anhydrase 9
CDDP	Cisplatin
CI	Confidence interval
CMF	Cyclophosphamide, 5 fluorouracil, methotrexate
CMT	Chemoradiation
CPR	Complete pathological response
CRM	Circumferential margin
CSS	Cause-specific survival
CT	Computerised tomography
DAB	Diaminobenzine tetrahydrochloride
DFS	Disease-free survival
DM	Distant metastases
DNA	Deoxyribonucleic acid
DPD	Dihydropyrimidine dehydrogenase
dUMP	Deoxyuridine monophosphate
ECL	Enhanced chemoluminescence
EDTA	Sodium ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMEMS	Eagle's minimal essential medium
EORTC	European Organisation for Research and Treatment of Cancer
5FU	5-Fluorouracil
5'-DFUR	Doxifluridine
FdUMP	5-fluoro-2'-deoxyuridine-5'-monophosphate
FdUTP	5-fluoro-2'-deoxyuridine-5'-triphosphate
FITC	Fluorescein Isothiocyanate
FUTP	5-fluorouridine-5'-triphosphate
HCL	Hydrochloric acid
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography analysis
HPV	Human papilloma virus

HRP	Streptavidin Peroxidase
IHC	Immunohistochemistry
IV	Intravenous
LR	Local recurrence
MMC	Mitomycin C
MRI	Magnetic resonance imaging
mTHF	5-10 methylenetetrahydrofolate
MVD	Microvessel density
NGS	Normal goat serum
NSABP	National Surgical Adjuvant Breast and Bowel Project
OS	Overall survival
PBS	Phosphate buffered saline
PCNA	Proliferating nuclear cell antigen
R0	Resection margin clear of carcinoma
R1	Microscopic disease at resection margin
R2	Macroscopic disease at resection margin
RFS	Relapse-free survival
RNA	Ribonucleic acid
RT	Radiotherapy
RTOG	Radiation Therapy Oncology Group
TBS + T	Tris buffered saline with tween
TME	Total mesorectal excision
TS	Thymidylate synthase
TP	Thymidine phosphorylase
UICC	International Union Against Cancer

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# **Chapter 1:**

## **General Introduction**

# **Chapter 1**

## **Section 1          Anal Carcinoma**

### **1.1                  Introduction**

Epidermoid cancer of the anus is relatively rare and accounts for less than 3% of all large bowel malignancies. It includes tumours of the anal canal and perianal skin i.e. tumours within a radius of approximately 5cm from the anal orifice.

In the United Kingdom approximately 700 new cases are registered annually. There are two peak incidences; one at 40-50 years and another at 70-80 years. There is a slight female preponderance overall. Cancers of the anal canal are three times more common in females, whereas cancers of the anal margin are commoner in males.

### **1.2                  Aetiology**

Human papilloma virus (HPV) infection is closely correlated with squamous cell anal carcinoma [1]. Using PCR, the presence of the HPV genome has been identified in 80-85% of cases; HPV type 16 was the most frequent type detected [2, 3]. This parallels the prevalence seen in cervical and vulval carcinoma in women [4].

Even before the current HIV epidemic, an excess risk of 40-50 times was observed in the homosexual population [5]. Immunosuppression is a further important risk factor, and anal cancer is not uncommon in renal and cardiac transplant recipients. There is also an increased risk associated with cigarette smoking. In a case-control study by Daling and colleagues [6], current cigarette smoking was a major risk factor in both sexes, relative risk 7.7 in women and 9.4 in men. This is similar to the report by Daniell [7] who noted that 54% of 13 women with anal cancer were current smokers compared to only 26% of 202 age-matched patients with colon cancer.

### **1.3                  Pathology**

Macroscopically the tumour arises close to the anal margin either in the skin or anal canal. Early appearances are often of a warty nodule or infiltrating area of ulceration. Microscopic appearances demonstrate that more than 90% are squamous, basaloid or cloacagenic carcinomas arising predominantly from the squamous epithelium. Less

than 5% appear to represent adenocarcinoma arising from the glandular mucosa of the upper anal canal, the anal glands and ducts and these are usually mucus secreting. Other less common tumours include anal melanomas, sarcomas and lymphomas.

Tumours arising at the anal margin tend to be well differentiated and keratinising akin to squamous skin cancers, whereas those arising in the anal canal are often poorly differentiated squamous cell carcinomas. Basaloid tumours arise in the transitional zone around the dentate line and constitute 30-50% of all anal canal tumours.

## **1.4 Clinical presentation**

The initial and most common symptom is bleeding and occurs in approximately 50% of patients [8-10] and 25% [10] present with an obvious mass and discomfort. Pruritus and discharge occur in 25% [10] and less commonly patients may present with faecal incontinence or a rectovaginal fistula. Diagnosis can be made on rectal examination.

Approximately one third of patients have enlarged inguinal lymph nodes but on biopsy only 50% will confirm metastatic spread. The remainder are caused by secondary infection. Biopsy or fine-needle aspiration is therefore recommended to confirm the involvement of inguinal nodes.

## **1.5 Staging and grading**

The TNM (Tumour-Node-Metastasis) staging system is most commonly used (UICC 1997); see Table 1.1 [11]. The TNM classification of anal cancers is primarily clinical, taking into account the fact that primary treatment is now a combination of chemotherapy and radiotherapy. Nodal status is based on distance from the primary site rather than the number of nodes involved, as this has more prognostic significance.

Tumours are graded according to a three-grade system, which takes into account the degree of anaplasia according to the nuclear to cytoplasmic ratio and nuclear frequency. They are graded as well, moderate or poorly differentiated.

**TNM Staging (UICC 5<sup>th</sup> Edition 1997)****T- Primary tumour (T)**

- TX     Primary tumour cannot be assessed
- T0     No evidence of primary tumour
- Tis     Carcinoma in situ
- T1     Tumour 2 cm or less in greatest dimension
- T2     Tumour more than 2cm but not more than 5cm in greatest dimension
- T3     Tumour more than 5cm in greatest dimension
- T4     Tumour of any size invading adjacent organ(s), e.g., vagina, urethra,  
Bladder

**N- Regional lymph nodes (N)**

- NX     Regional lymph nodes cannot be assessed
- N0     No regional lymph node metastasis
- N1     Metastasis in perirectal lymph node(s)
- N2     Metastasis in unilateral internal iliac and/or inguinal lymph node(s)
- N3     Metastasis in perirectal and inguinal lymph nodes and/or bilateral  
internal iliac and/or inguinal lymph nodes

**M- Distant metastasis (M)**

- MX     Presence of distant metastasis cannot be assessed
- M0     No distant metastasis
- M1     Distant metastasis

**Table 1.1** The TNM staging system for anal carcinoma

## **1.6 Pattern of spread**

The primary tumour usually grows in an annular fashion extending through the wall of the anal canal to involve the perianal tissue or rectum. It may also spread outwards into the sphincters, the rectovaginal septum and the vagina. Lymph node metastases are found initially in the perirectal group of nodes and thereafter in inguinal, haemorrhoidal and lateral pelvic lymph nodes.

Anal margin tumours are more likely to involve inguinal nodes than anal canal tumours. 30% of patients will have involvement of their inguinal nodes, however in early T1/T2 tumours the rate of involvement is approximately 12% [8-10]. It is usually unilateral and occasionally bilateral but never contralateral to the tumour.

The pelvic nodes will almost always also be involved particularly with increasing tumour stage and in poorly differentiated tumours. When the rectum is directly involved, the cancer may spread via the inferior mesenteric lymph nodes. The overall incidence of pelvic lymph node metastases is in the region of 25-30% [10].

The prognosis is much worse with synchronously involved nodes than with metachronous spread. Haematogenous spread tends to occur late and is usually associated with advanced local disease. The principal sites of metastases are the liver and lung, however at presentation <5% have spread to these sites [10, 12, 13]. Metastases have also been described in the kidneys, brain and adrenal glands.

## **1.7 Treatment**

Up until the mid 1980's surgery was the first line treatment for anal carcinomas. An abdominoperineal excision was commonly performed necessitating a permanent colostomy. However, non-surgical radical treatment with combined chemotherapy and radiotherapy has become the treatment of choice in most cases. Following early work by Nigro et al [14] chemoradiation has become the standard treatment. Chemoradiation allows preservation of anorectal function with survival rates similar to those of surgery. In a series of non-randomised prospectively designed protocols, Cummings et al [15] established that the combination of 5-fluorouracil (5FU) and

mitomycin C (MMC) given concurrently with radiotherapy was more effective than 5FU and radiotherapy alone.

Three phase III trials addressed the issue of combining radiotherapy (RT) with 5FU and MMC [16-18]. The two European trials, UKCCR and EORTC, looked at the addition of the two chemotherapy agents to radiotherapy whilst the US RTOG trial investigated the role of adding MMC to 5-FU and radiotherapy. All trials used a continuous four or five day infusion of 5-FU in the first and last weeks of radiotherapy.

### **1.7.1 ACT I trial**

The UK Anal Cancer Trial (ACT I) [16], undertaken by the UK Co-ordinating Committee for Cancer Research (UKCCR) was the largest trial with 585 patients. It randomised patients to split-course radiotherapy alone or chemoradiotherapy with 5FU and MMC, see Figure 1.1. The 5FU was given as 1000mg/m<sup>2</sup> over 24 hours days 1-4 and 29-32 of radiotherapy

ACT I had a median follow-up of 42 months and it demonstrated a 46% reduction (95%CI 0.42-0.69,  $\chi^2=24.6$ ,  $p<0.0001$ ) in the risk of local treatment failure using CMT (5FU + MMC + RT) over that achieved by radiotherapy alone in patients with anal cancer. There was also a reduced risk of death from anal cancer (RR=0.71, 95%CI 0.53-0.95;  $\chi^2=5.4$ ,  $p=0.02$ ) and a non-significant overall survival advantage (RR=0.86, 95%CI 0.67-1.11,  $\chi^2=1.3$ ,  $p=0.25$ ).

Analysis of the parallel quality of life (QOL) study showed that the addition of chemotherapy did not impair QOL [19].

### **1.7.2 RTOG and EORTC trials**

The European Organisation for Research and Treatment of Cancer (EORTC) [17] trial of 110 patients also demonstrated similar results to ACT I. The trial run by the Radiation Therapy Oncology Group (RTOG) [18] demonstrated an advantage for the addition of MMC to 5-FU and radiotherapy, improving the disease-free survival (DFS) at 5 years from 50 to 67% ( $p<0.003$ ). In a series of non-randomised



prospectively designed protocols, Cummings et al [15] also established that the combination of 5FU and MMC given concurrently with radiotherapy was more effective than 5FU and radiotherapy alone.

As a result of these trials, the current standard treatment for patients with epidermoid anal carcinoma is a combination of RT + 5-FU + MMC, with surgery reserved for those who fail on this regimen, thus saving the majority of patients from the necessity of living with a colostomy.

## **1.8 Current research**

The results of ACT I showed that patients with anal carcinoma have a relatively poor prognosis; 46% (265/577) had a local treatment failure and 50% were dead at 5 years. Thus further effort is necessary to optimise treatment to improve local control and survival rates. Current research is concentrating on several different approaches; including modification of the radiotherapy schedule; changing the chemotherapy regimen given concurrently with radiotherapy and administering additional courses of chemotherapy. Another promising area of research is whether it is possible to determine which pre-treatment factors are predictive of patients' responses to chemoradiation.

### **1.8.1 ACT II trial**

This trial employs a continuous course of radiotherapy, unlike the trials described previously, which all involved gaps at different time points during the schedule. ACT II also investigates the role of cisplatin in combination with 5FU and radiotherapy. Cisplatin has been found to be more effective than MMC in other squamous cell tumours [20-22], and phase II trials in anal carcinoma have demonstrated a higher response rate with lower colostomy rates [23]. ACT II also incorporates a further randomisation to additional chemotherapy with cisplatin and 5FU at the end of chemoradiation, to assess whether maintenance chemotherapy will benefit patients in terms of DFS, see Figure 1.2.

## **1.9 Conclusion**

Anal cancer is one of the few disease sites where chemoradiation has made a significant impact on treatment. A large proportion of patients can now be spared a permanent colostomy and retain sphincter function. Further research is needed to maximise the benefit which can be gained from this combined modality treatment. Some authors dispute the benefit of chemoradiation over surgery alone for small T1 tumours. In contrast, the results of chemoradiation in larger T3 and T4 cancers remain poor. Many surgeons advocate either surgery alone or a combination of preoperative chemoradiation followed by surgery.

The present study aims to look at the potential role of molecular markers in predicting response and outcome to treatment. It also aims to look at the role of capecitabine, a new and exciting oral 5FU agent, in which chemoradiation phase II trials in anal cancer are currently underway. This chemotherapy has the potential to improve therapeutic benefit by selective conversion to 5FU within the tumour cells and not normal tissues.

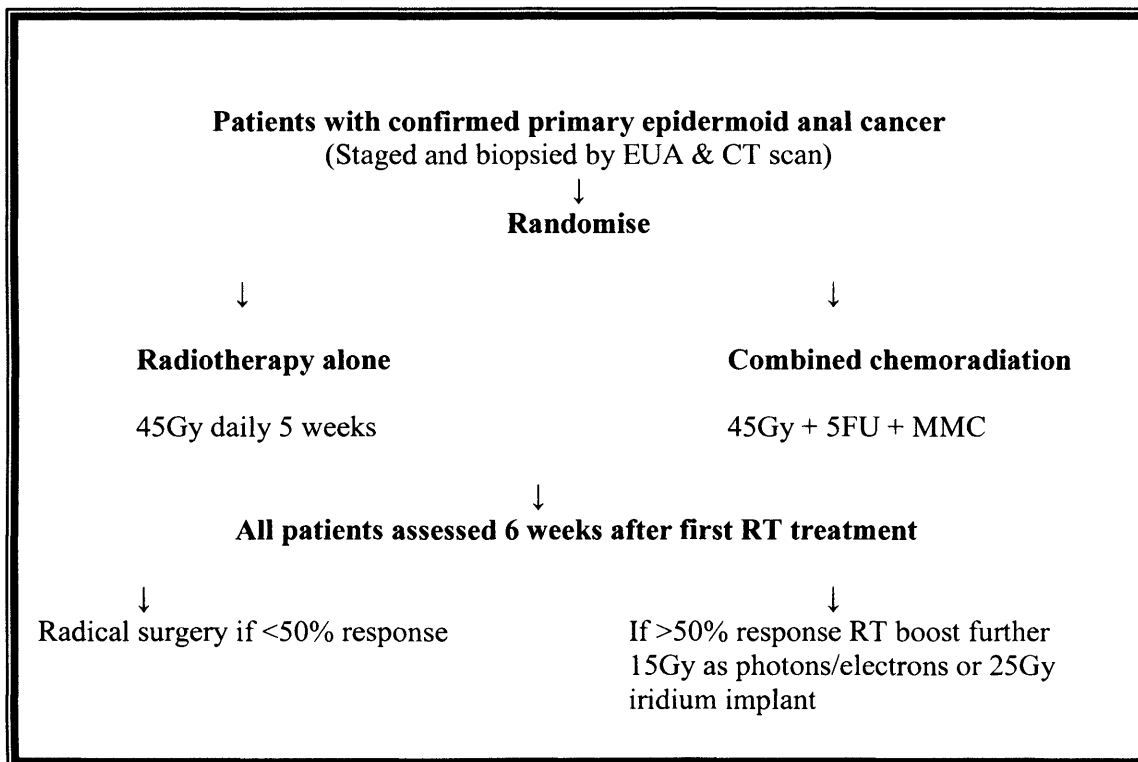


Figure 1.1 ACT I algorithm

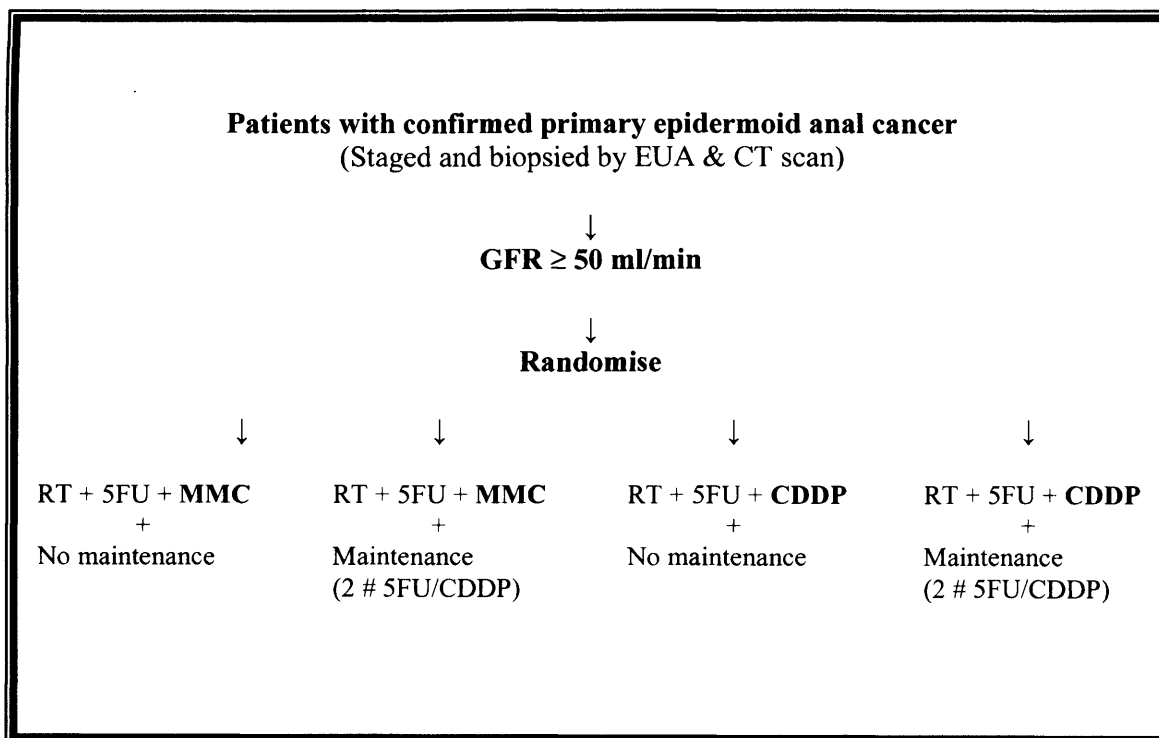


Figure 1.2 ACT II algorithm

## **Section 2            Rectal Carcinoma**

### **1.10            Introduction**

Colorectal carcinoma is one of the commonest cancers in Western countries and the fourth commonest cancer worldwide. Despite recent advances in early diagnosis and adjuvant therapy; this disease is still associated with a high mortality [24]. There are 28,000 new cases a year in the United Kingdom and 20,000 deaths (CRC fact sheet). Men and women are affected equally and incidence increases with age. Cancers of the rectum and recto-sigmoid junction account for approximately 50% of all colorectal carcinomas.

### **1.11            Aetiology**

#### **1.11.1           Environmental factors**

Colorectal cancer is more common in westernised countries and for this reason it is often termed an 'environmental' disease. A high intake of dietary fat and meat has been linked to an increased incidence [25] and dietary fibre has been proposed as accounting for the differences in the rates of colorectal cancer between Africa and westernised countries. This is on the basis that the increased intake of fibre may increase faecal bulk and reduce transit time through the bowel [25].

There is evidence to support an association between higher physical activity and a reduced risk of colorectal cancer; however the data currently available does not show a consistent association between obesity and an increased risk in this disease. There is evidence however, which suggests there is an association between obesity and an increased risk of developing adenomas [26], a common precursor of sporadic colorectal carcinomas. The use of hormone replacement therapy in women also appears to have some protection against the development of colorectal cancer and the risk seems lowest among long-term users. The risk appears to halve with 5-10 years use [27]. However it is not clear if this is a true causal association or a selection factor.

#### **1.11.2           Sporadic colorectal adenomas**

More than 70% of colorectal cancers develop from sporadic adenomatous polyps, and post-mortem studies have shown the incidence of adenomas to be 30-40% in Western

populations. Polyps are asymptomatic in the majority of cases and are often multiple. A number of genetic alterations, occurring as part of a sequence [28, 29], have been proposed to explain the development of a colorectal carcinoma from an adenoma [30].

### **1.11.3 Family history**

Recognised hereditary conditions account for approximately 5% of all colorectal cancers. The commonest are familial adenomatous polyposis (FAP) and hereditary non-polyposis colon cancer (HNPCC). Patients with these conditions usually have a family history of colorectal cancer presenting at a much earlier age than expected. Both FAP and HNPCC show dominant Mendelian inheritance.

In the case of FAP there is a germline mutation in the tumour suppressor gene for adenomatous polyposis coli (APC) on chromosome 5. If DNA is available from an affected individual, mutation detection is possible in about 70% of families. In HNPCC germline mutations in DNA mismatch repair enzymes occur. Five genes have now been identified; hMSH2, hMLH1, hPMS1, hPMS2 and GTBP. If both copies of the genes are mutated the cell and all its daughter cells are missing a vital mechanism for DNA repair.

### **1.11.4 Ulcerative colitis**

Patients with ulcerative colitis have a 2-8.2 relative risk of developing colorectal cancer compared to the general population, accounting for about 2% of colorectal cancers [31]. The risk increases with the duration of the colitis and the extent of disease is also important. Involvement of the right and transverse colon has a higher risk of developing colon cancer [31].

## **1.12 Pathology**

Colorectal cancers can be exophytic or fungating, or tumours may be ulcerated. More than 90% are adenocarcinomas. Tumours are generally classified according to their grade and histological subtype. See Table 1.2. The grading system is a combination of the degree of glandular formation, as well as cytological and nuclear features.

<u>Histological sub-type</u>	<u>Grade</u>
Mucinous adenocarcinoma	Well
Signet-ring adenocarcinoma	Moderate
Adenosquamous carcinoma	Poor
Squamous carcinoma	
Small cell carcinoma	
Choriocarcinoma	
Medullary carcinoma	

**Table 1.2** World Health Organisation Classification of malignant primary tumours of the large intestine

Most colorectal cancers are moderately differentiated, gland-forming adenocarcinomas. The less common sub-types are classified on the basis of the predominance of an unusual pattern as compared with the usual adenocarcinoma of the colon. Mucinous or colloid carcinomas exhibit the majority of tumour in mucin pools, which are often of low cellularity. Signet ring tumours display a large amount of intra-cellular mucus pushing the nucleus to the side of the cell. These are often associated with diffuse intramural spread beyond the obvious mucosal lesion. Less common colorectal tumours include carcinoid, small cell carcinomas and sarcomas.

### **1.13 Clinical presentation**

The symptoms at presentation depend significantly on the site of disease. Colon carcinomas may present simply as a change in bowel habit, unexplained weight loss or an iron deficiency anaemia. If the tumour is annular or exophytic, obstruction may occur resulting in pain, abdominal distension and vomiting. In the case of rectal carcinomas the commonest presentation is rectal bleeding, which may be

accompanied by pain and tenesmus. Partial or complete obstruction may also occur in 5-15% of rectal tumours, and if it occurs, it is associated with a poorer survival [32].

### **1.14 Staging and diagnosis**

The first widely used staging system for colorectal cancer was introduced by Duke in the 1930's. This is still commonly used but the preferred system is often the TNM classification. This is a unified staging system from the UICC (International Union Against Cancer) and AJCC (American Joint Committee on Cancer) [11]. It includes accounting for the number of positive lymph nodes, which is an important prognostic factor, Tables 1.3 and 1.4. The new TNM classification calls for at least 12 lymph nodes to be examined.

Any patient with a clinical history suggesting colorectal cancer should undergo examination of the entire colon. The aim is to diagnose the primary tumour and exclude any synchronous polyps or cancers. This is commonly done by colonoscopy or barium enema. Metastases should be excluded either by CT or chest X-ray and liver ultrasound. In the case of rectal carcinoma, a pre-operative CT, see Figure 1.3, or preferably an endorectal ultrasound or MRI may also be performed to try and accurately stage the primary tumour. This is necessary as pre-operative treatment is now common in locally advanced rectal tumours.

### **1.15 Pattern of spread**

Histological stage of spread at diagnosis is the most important prognostic variable. The depth of mural invasion is one of the most important prognostic factors. In colon carcinoma, the mesentery and serosal surfaces are at greatest risk for local tumour penetration. However in rectal cancer perirectal fat and adjacent organs are most commonly involved by direct invasion through the bowel wall. This difference in local spread between the two sites is extremely important in terms of treatment and prognosis. If perineural invasion has occurred local recurrences are more common.

The degree of lymph node involvement correlates with the depth of tumour and involvement occurs in almost 50% of those with deep tumours. Approximately 10-

**TNM Staging (UICC 5<sup>th</sup> Edition 1997)****T- Primary tumour (T)**

TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
T1	Tumour invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades through muscularis propria into subserosa, or into non- peritonealised pericolic or perirectal tissues
T4	Tumour directly invades other organs or structures, and/or perforates visceral peritoneum

**N- Regional lymph nodes (N)**

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1 to 3 regional lymph nodes
N2	Metastasis in 4 or more regional lymph nodes

**M- Distant metastasis (M)**

MX	Presence of distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

**Table 1.3** The TNM staging system for colorectal carcinoma



<b><u>Stage Grouping</u></b>				
<b>TNM</b>			<b><i>Dukes</i></b>	
Stage 0	Tis	N0	M0	
Stage I	T1	N0	M0	A
	T2	N0	M0	
Stage II	T3	N0	M0	B
	T4	N0	M0	
Stage III	Any T	N1	M0	C
	Any T	N2	M0	
Stage IV	Any T	Any N	M1	D

**Table 1.4** TNM stage grouping**Figure 1.3** CT demonstrating a carcinoma of the rectum

15% of all colorectal cancer patients will present with metastatic disease and the commonest sites are liver and lung followed by adrenals, ovaries and bone. The colon drains into the portal venous system directly to the liver, whereas the rectum has a dual system. The superior haemorrhoidal veins enter the portal system to the liver, whereas the middle and inferior haemorrhoidal veins drain to the inferior vena cava and then spread to the lungs via the systemic circulation. Lung metastases are therefore found more commonly in patients with cancers of the lower rectum.

## **1.16 Treatment of rectal cancer**

Local recurrence (LR) is a frequent problem in rectal cancer and this is reflected in the current treatment strategies for this disease. LR is an important cause of mortality and morbidity, and occurs in 10-60% of rectal cancers even after a curative resection [33, 34], with a median time to recurrence of twelve months. When local failure does occur, it is severely debilitating and salvage therapy has been of limited success. Most local recurrences are isolated and unaccompanied by disseminated disease. This is in contrast to colonic carcinoma where LR is rare and usually accompanied by disseminated disease [35].

Surgical resection is the treatment of choice in patients with localised rectal cancer. It potentially offers the chance of cure. However, more than half of all patients with rectal cancer will die of metastatic disease and this includes up to 20% of patients with apparent early stage tumours [32].

Two major factors influence local recurrence in rectal cancer. Surgery-related factors include the type of surgery performed, for example total mesorectal excision (TME) or a low anterior resection. The extent of lymphadenectomy, presence of post-operative anastomotic leakage and inadvertent tumour perforation during operation are also important factors for local recurrence [36]. Tumour-related factors include anatomical location, histological subtype, tumour grade, status of circumferential margin and presence of neural, venous or lymphatic invasion [37].

Approximately 20% of patients with rectal cancer will present with locally advanced tumours, which are partially or totally fixed (T3/T4). Standard surgical resection in

these patients is associated with high rates of incomplete excision and later local recurrence. Tumour fixation is recognised as one of the most important pre-treatment factors for predicting the likelihood of achieving a curative resection as well as achieving good overall and disease-free survival [38]. After surgery, tumour located 1mm or less from the radial margin of the excision is usually considered an incomplete resection (R1). This is associated with a high risk of local recurrence.

It is therefore important to achieve a curative surgical excision in the treatment of rectal cancer. For early tumours, surgery may be all that is required and in recent years major advances have been made in the surgical treatment of rectal cancer. In the UK the majority of patients will now undergo a total mesorectal excision (TME). However, in the case of more locally advanced tumours, surgery may be preceded by chemoradiation.

### **1.16.1 Total Mesorectal Excision**

A significant number of patients with rectal carcinoma will have full-thickness penetration of the bowel wall or involvement of mesorectal lymph nodes. The mesorectum is the lymphovascular, fatty and neural tissue that is circumferentially adherent to the rectum. The rectum and potentially affected mesorectum should therefore be considered as a single entity. TME results in the removal of the carcinoma and mesorectum en bloc. The aim is to reduce the incidence of positive post-operative margins and therefore, local recurrence.

Several series have shown a decrease in local recurrence rates following TME surgery, with LR rates reduced to 5-8% [39, 40]. A recent study by Kapiteijn [41], demonstrated that pre-operative radiotherapy in addition to TME reduced the risks of local recurrence further. The rate of local recurrence at two years was 2.4% in the radiotherapy-plus-surgery group and 5.3% in the surgery-only group.

### **1.16.2 Neoadjuvant treatment**

#### **1.16.2.1 Short course radiotherapy**

Currently, in resectable rectal cancer there are two choices for the delivery of adjuvant radiotherapy, either pre-operative or post-operative. Pre-operative

radiotherapy has advantages of producing less morbidity with better compliance rates. Since the publication of the Swedish Rectal Cancer Trial, which revealed a significant improvement in local control and survival with intensive short-course preoperative radiation, some physicians have advocated this radiation schedule [42]. A recent meta-analysis of 14 trials also revealed a significant improvement in survival with intensive short-course preoperative radiation [43]. Typically, the intensive short-course includes 25Gy in five daily fractions followed by surgery one week later. Although a survival benefit was demonstrated this schedule has been associated with some morbidity and it does not enhance sphincter preservation. It also does not lead to significant downstaging and therefore is not ideal for the more locally advanced rectal tumours. In a study by Marijnen [44] a decrease in tumour size and the number of recovered lymph nodes was observed but there was no change in tumour or lymph node classification.

#### **1.16.2.2 Pre-operative chemoradiation**

Between 20-30% of cases of rectal cancer present as locally advanced tumours, which are either fixed or tethered because of direct invasion to other pelvic organs, the pelvic sidewall or sacrum. This is a particular problem in males with anterior tumours where there is little, if any surgical plane between the rectal cancer and the prostate. Fixity has been shown to predict the likelihood of a non-curative resection [45, 46].

Surgical resection in these patients is associated with high rates of incomplete excision and later local recurrence. In this group of patients the aim is to downstage the tumour rendering it resectable. The results with radiotherapy alone, at doses of 40-50Gy, do show increased resectability rates [47, 48] with reduced rates of LR [47]. However, when chemotherapy and radiotherapy are synchronously combined further downstaging may be achieved [49]. The rationale for chemoradiation is that it combines systemic treatment simultaneously with a loco-regional treatment. In addition there are synergistic or additive effects between the two modalities. When compared to surgery alone and surgery plus post-operative radiotherapy, the addition of concomitant and adjuvant chemotherapy to radiotherapy in the post-operative setting has shown improved results in local control and survival [50, 51]. However, the value of adding radiotherapy to chemotherapy in the post-operative setting is not

so clear. In the NSABP R-02 (National Surgical Adjuvant Breast and Bowel Project) trial, patients were randomised to post-operative chemotherapy with or without radiotherapy. The radiotherapy led to a significant reduction in local relapse rates but the incidence of distant relapse and hence survival was unchanged [52]. This suggests that only those patients deemed at high risk of local recurrence should be offered post-operative chemoradiation.

Pre-operative chemoradiation produces less morbidity and better compliance rates than in the post-operative setting. It is clear pre-operative chemoradiation can lead to significant downstaging of the tumour [49, 53]. In the study by Rich et al [53], a complete pathological response was achieved in 29% with infusional 5FU and radiotherapy. Others have also confirmed favourable resectability rates for fixed cancers and demonstrated improved local control, when 5FU is combined with radiation [54, 55]. One study demonstrated a 100% 10 year survival for patients achieving a complete pathological response (CPR) following pre-operative radiotherapy alone [56].

For patients with clinically resectable disease, the preoperative approach is commonly used in situations where, at initial presentation, sphincter-preserving surgery is not technically possible. In tumours that are likely to have an R1 resection, pre-operative chemoradiation is given to downstage the primary tumour so that an R0 resection rate is more likely to be achieved. However, even with this pre-operative approach the overall five year survival rate for this group of patients is still low at 20-30%, despite a curative resection [57] and there is still a high risk of local recurrence [54].

### **1.17 Current research**

Despite recent improvements in surgery and pre-operative radiotherapy and chemotherapy, the survival remains poor for those patients with locally advanced carcinoma of the rectum. Future research into the newer chemotherapy drugs, oxaliplatin and irinotecan, alone or in combination with radiotherapy is needed. The main strategies for improving outcome in rectal cancer must address the following issues; ensuring a curative surgical resection, reducing the risk of metastatic disease and what is the most effective adjuvant chemotherapy.

## **Section 3            5FU-based chemoradiation**

### **1.18            Introduction**

For many years the combination of chemotherapy and radiotherapy has constituted one of the main avenues for research in oncology. The biological mechanisms of combination therapy are complex and not fully elucidated. Used alone or in combination with surgery, chemoradiation is rapidly becoming the gold standard for the treatment of certain tumours, for example anal and locally advanced rectal carcinomas. This is due to observed improvements in local control in particular but also survival.

To maximise the benefit from the combination of these two treatment modalities it is important to understand the nature of how the drugs and radiation interact.

### **1.19            Rationale for combined chemotherapy and radiotherapy**

The purpose of combining radiation and chemotherapy is to achieve a therapeutic gain by increasing the effect on the tumour without concomitantly enhancing critical normal tissue reactions. The mechanisms of interaction between chemotherapy and radiotherapy have been divided into four generic types by Steele [58].

1. **Spatial Co-operation**: each modality treats a different anatomical site e.g. systemic adjuvant chemotherapy and local radiotherapy in breast cancer.
2. **Independent cell kill**: agents act independently of each other and produce a greater effect than either agent alone.
3. **Protection of normal tissue**: the tolerance of the dose-limiting normal tissue can be modified allowing a higher dose of the treatment modality to be used e.g. the use of amifostine as a radio-protector.
4. **Enhancement of tumour response**: agents may sensitise tumour cells to the effects of another treatment modality. The interaction may therefore be additive, supra-additive or sub-additive.

Another purpose for using combined modality treatment is that it may overcome sub-clones of cells in tumours with increased resistance to drugs and radiotherapy. These sub-clones may have a selective advantage and their survival and growth may lead to treatment failure. This resistance to treatment might be reduced through the combined use of radiation and drugs, provided the mechanisms of resistance are independent. For example radiation failure is often related to radio-resistant hypoxic cells in areas of poor vascularity. Mitomycin C is an established chemotherapy agent which, under hypoxic conditions functions as an alkylating agent. Co-administration of this agent with radiation may overcome the problem of radio-resistant hypoxic cells.

Achieving a therapeutic gain can be difficult because one of the major limitations is that of selectivity of the treatment to the tumour cells and not normal tissues. The combination of treatments must take this into account, as the toxicity of normal tissues is invariably the dose-limiting factor for both of these modalities when used alone; in combination this toxicity may be much more severe. A small survival benefit may not justify a significant increase in toxicity by using a combination schedule.

When analysing the results of studies using chemoradiation and for future research into the mechanisms of interaction, Coleman and colleagues [59] have suggested the following considerations:

1. What is the target of each agent? *e.g.* DNA, RNA, enzyme, cell receptor
2. Is the target stable? *e.g.* cell cycle variation, tumour heterogeneity, drug resistance, hypoxia
3. Can the target be reached?
4. What is the optimum schedule?
5. What are the combined toxicities?

It is important to determine the optimum timing and dose of each modality to achieve the most effective schedule, with acceptable normal tissue toxicity. *In vitro* and *in vivo* research can help define the mechanisms of interaction between each modality,

facilitating the development of clinical schedules. In current clinical practice there are several variations of radiotherapy and chemotherapy combinations:

1. **Sequential**: one modality following completion of the other.
2. **Concurrent**: both modalities are given at the same time.
3. **Alternating**: each modality is divided into blocks and alternated.

Most patients' die from metastatic disease and therefore combined modality treatment, as well as improving local control, must also target tumour dissemination. As a consequence of this, the concept of adjuvant and neoadjuvant treatment has emerged. This strategy targets micrometastases at an early stage in the patient's treatment and an example would be neoadjuvant 5FU-based chemoradiation in locally advanced rectal cancer.

## 1.20 5-Fluorouracil

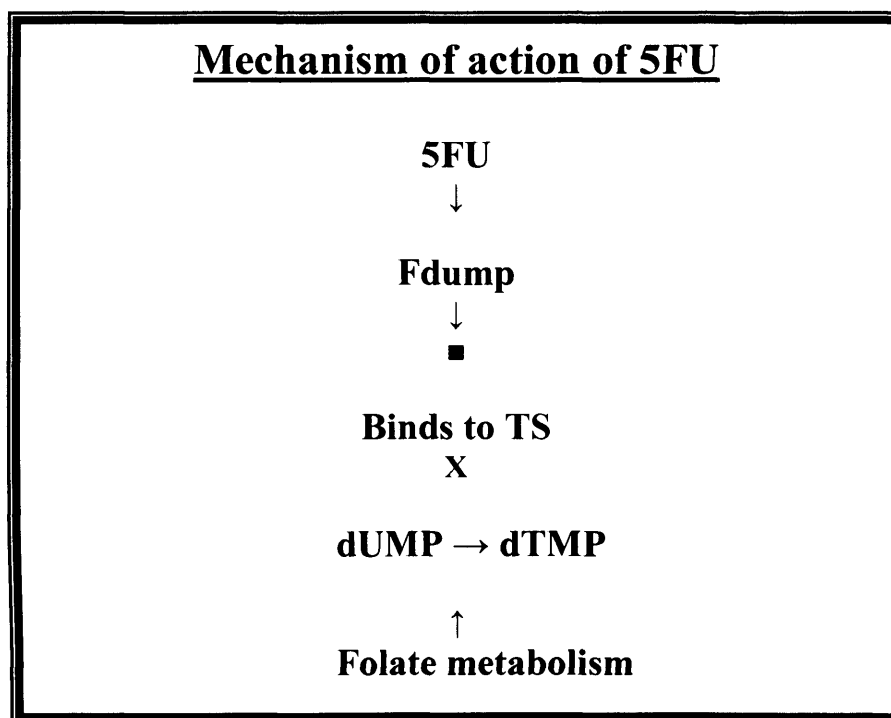
### 1.20.1 Mode of action and sensitivity

5-fluorouracil is a prodrug that is subject to both catabolism and anabolism. 80% of the dose is catabolised and inactivated in the liver by dihydropyrimidine dehydrogenase (DPD) [60]. An additional 15-20% is excreted in the urine [60].

5FU is an analogue of uracil and during anabolism a series of phosphorylation reactions lead to the formation of 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), 5-fluoro-2'-deoxyuridine-5'-triphosphate (FdUTP), and 5-fluorouridine-5'-triphosphate (FUTP), the active metabolites of fluorouracil. The cytotoxic effects of fluorouracil are thought to result from the binding of 5-FdUMP to thymidylate synthase (TS), which is the enzyme responsible for converting deoxyuridine monophosphate (dUMP) to thymidylate (dTMP), a necessary step in the synthesis of DNA, see Figure 1.4. This results in the inhibition of DNA synthesis and apoptosis with a depletion of thymidine nucleotides and an accumulation of deoxyuridine nucleotides. This leads to changes in other nucleotide pools [61, 62], arrest of cells in S phase of the cell cycle (growth arrest), and ultimately to DNA damage and fragmentation. However, it must be remembered that a salvage pathway exists as demonstrated *in vivo*, in which nucleosides are brought into the cell and used for



DNA and RNA synthesis. This allows the bypass on any endogenous block and may, in part, explain resistance to 5FU [63].



**Figure 1.4** Mechanism of action of 5FU

As well as inhibition of TS, FdUMP can also be converted to fluorodeoxyuridine triphosphate (FdUTP) and become incorporated into DNA [64]. However, this route of incorporation into DNA does not seem necessary for cell death to occur [65]. 5FU can also kill cells by RNA-dependent mechanisms. 5FU can be metabolised to fluorouracil monophosphate (FUMP) and then ultimately to fluorouracil triphosphate (FUTP) which can be incorporated readily into RNA in preference to UTP; this affects the production of ribosomal RNA [66]. It also interferes with messenger RNA function including transcription [67], translation [68] and splicing [69].

Sensitivity to 5FU is determined by a number of factors. Recent studies have suggested that patients with low primary tumour levels of TS expression or TS messenger RNA (mRNA) have a superior overall response to the drug and better survival rates [70, 71]. Response rates to 5FU can be increased by combination with

leucovorin [72]. Leucovorin acts by increasing and prolonging the competitive inhibition of TS by stabilising the ternary complex formed by the active metabolite of fluorouracil [73]. The size of the intracellular pool of reduced folate is also important in determining 5FU sensitivity. Improved response rates to 5FU have been seen in head and neck cancer patients with high intra-tumoural levels of reduced folates [74]. DPD is the primary rate-limiting enzyme in the catabolism of 5FU and a low level of DPD is correlated with a higher likelihood of tumour response [75] due to the resulting higher levels of 5FU. With the introduction of capecitabine (Xeloda), an oral 5FU analogue, expression of thymidine phosphorylase has also become important as this enzyme catalyses the final conversion of the drug to 5FU [76].

Since the 1960's 5FU has remained the most extensively used chemotherapeutic agent in colorectal cancer. It is used in the adjuvant setting at present as a single agent. In the metastatic setting 5FU has been combined with other cytotoxic agents to enhance response rates. These regimens are associated with increased toxicity. Oxaliplatin and irinotecan are currently the two chemotherapy agents, which are attracting the most interest in colorectal cancer and in the case of anal cancer, MMC and cisplatin. 5FU is also commonly used in the treatment of upper gastrointestinal tumours particularly in the metastatic setting and in chemoradiation protocols. It can be administered either as an intravenous bolus (IV) or in an infusional form. The dose limiting toxicities vary with the mode of administration. In the case of IV bolus, immune suppression is the major toxicity and with infusional it is diarrhoea and the 'plantar-palmar' syndrome.

## **1.20.2 5-Fluorouracil as a radiosensitiser**

In addition to being cytotoxic, 5FU also acts as a radiosensitiser [77, 78] and has become one of the agents most widely used to sensitise tumour cells to the effects of radiation in clinical practice. As a radiosensitiser it is likely to be most effective when given concurrently with radiation.

### **1.20.2.1 Possible mechanisms of radiosensitisation**

FdUrd (5-fluoro-2'-deoxyuridine) is an analogue of deoxyuridine and is also used as a chemotherapeutic agent. It differs from 5FU in that it primarily has DNA effects and

it is metabolised directly to FdUMP by phosphorylation (via thymidine kinase). It has been shown that FdUrd is a potent radiosensitiser of HT29 cells [79] and it was more effective when drug treatment preceded radiation compared with following radiation. The degree of sensitisation correlated with the level of TS inhibition [79] and depletion of dTTP pools [61] and was blocked by co-incubation with thymidine [79]. There was also a decrease in double-strand break and sub-lethal damage repair [61, 64].

Three hypotheses have been generated to account for fluoropyrimidine-induced radiosensitisation:

1. **Nucleotide pool perturbations:** The pool of nucleotide substrates necessary for the repair of radiation induced DNA damage is depleted [78]. This depletion compromises the ability of polymerases to find the correct base for repair, leading to misrepair, single strand breaks (SSB) and double strand breaks (DSB). However the dTTP pools are depleted within 1-2 hours of drug exposure whereas radiosensitisation takes several hours to develop. Therefore pool changes alone do not solely account for radiation sensitivity.

2. **Cell cycle redistribution:** Treatment with 5FU arrests cells in S phase and blocks cells that are not in S phase at the G1/S phase interface. It has been suggested that early S phase is a sensitive phase to radiation [62], and this accounts for the radiosensitisation effect. Work by Lawrence et al [80] showed that HT29 cells exposed to FdUrd generated significantly higher levels of cyclin E-dependent kinase activity. Cyclin E is responsible for driving cells into early S phase. However, some studies have shown that 5FU can sensitise cells after radiation. Byfield [81] found that in Hela and HT29 cells, 5FU radiosensitisation only occurred when drug exposure followed radiation. In colorectal cancer cell lines marked differences are not apparent in radiation sensitivity between different phases of the cell cycle [82]. Therefore cell cycle effects also do not account fully for the radiosensitisation effect.

3. **Incorporation of FdUTP into DNA:** This does not appear to be a requirement for radiosensitisation. This is based on results of experiments with TS inhibitor CB3717.

This inhibitor cannot be incorporated into DNA and yet it is an excellent radiosensitiser in HT29 cells [82].

**4. Increased intra-cellular retention of 5FU and its metabolites:** This results from radiation exposure and has recently been demonstrated *in vivo* by Blackstock [83]. This study examined HT29 colon cancer xenografts in athymic, nude mice. One group received 5FU alone and a second group received 5FU after a 10Gy dose of radiation. Spectroscopic analysis was then performed. The tumour retention of 5FU was more prolonged in the group receiving radiation before the drug infusion. This is consistent with the hypothesis that radiation is potentiating the cytotoxic effects of 5FU.

These are all possible mechanisms for 5FU radiosensitisation but clearly no one mechanism is responsible. Lawrence et al [78] have proposed that, rather than taking the viewpoint that 5FU sensitises cells to the effects of radiation, it is reasonable to consider that radiation may potentiate the cytotoxic effects of 5FU. This may explain the differences seen between different schedules. Radiosensitisation appears to take place under conditions that produce some cytotoxic effect from 5FU alone [62, 79, 81]. This would suggest that the cytotoxic effect from 5FU is important in the mechanism of cell radiosensitisation. Byfield [63] determined that the extent of radiosensitisation was dependent on the duration and concentration of exposure to the drug as well as sensitisation occurring only when 5FU is administered at cytotoxic doses.

It is clear to see that the mechanisms of interaction between 5FU and radiation still remain elusive. As a result, determining the appropriate scheduling of the two treatment modalities is difficult. Administering 5FU as a continuous infusion overcomes some of these difficulties of scheduling and is thought to increase the exposure of cells in S phase to the drug. The limitations of drug exposure are that only 3-25% of colorectal cancer cells are actively synthesising DNA at any moment in time and, because TS inhibition after administration of bolus doses of 5FU is relatively short-lived (half-life approx 10 minutes), the fraction of colorectal cancer cells susceptible to bolus 5FU is small [73]. However, even if the results are superior, the use of protracted infusional 5FU given with radiotherapy is not an approach that is

currently feasible in many UK centres because of the cost and additional staffing requirements.

### 1.20.3 Capecitabine (Xeloda)

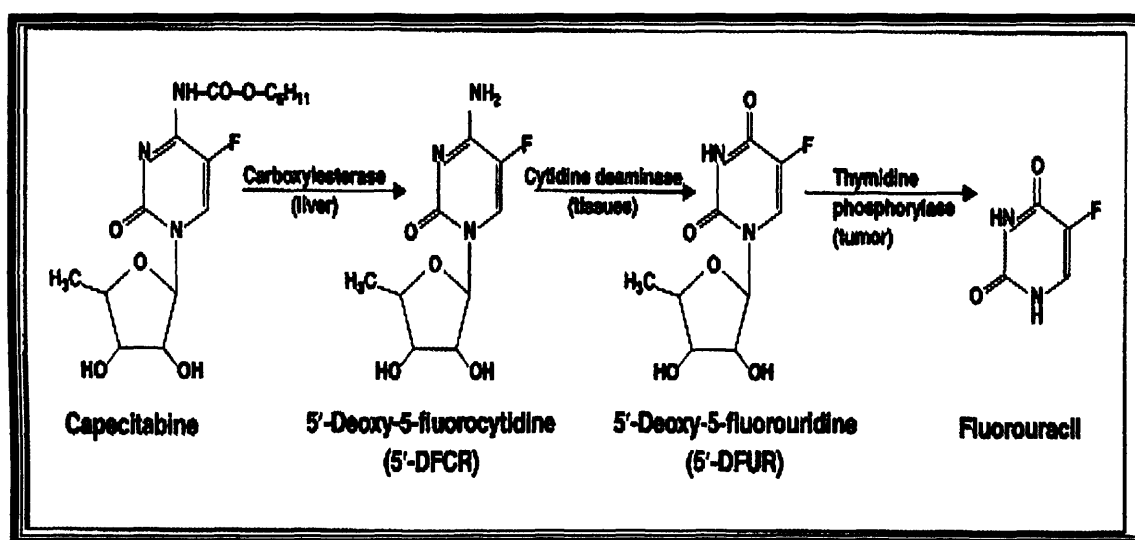
5FU is one of the main chemotherapies used alone or in combination, in the systemic treatment of solid cancers and it forms the mainstay of treatment in colorectal tumours. 5FU is given in intra-venous form and is associated with morbidity, which limits the intensity and duration of treatment; these include diarrhoea, stomatitis, neurological and myelosuppressive toxicity [84]. Although protracted intravenous infusion provides a continuous exposure to 5FU and limits the problems of cell cycle specificity and short half-life of bolus 5FU, it still has associated toxicity requires the insertion of a central venous catheter.

The development of an oral form of 5FU has several important advantages including patient convenience and reduced cost. One of the first oral forms of 5FU to show promise was Doxifluridine (5'-DFUR). This was a prodrug with preferential conversion to 5FU at the tumour site, by exploiting the higher levels of pyrimidine nucleoside phosphorylase (PyNPase) found in tumour tissue compared to normal tissue [85]. It showed good bioavailability after oral administration. The major disadvantage however was its dose-limiting toxicity, principally diarrhoea, which was as a result of 5FU generation by PyNPase, in the gastrointestinal tract [86].

Capecitabine (Xeloda, N4-pentoxycarbonyl-5'-deoxy-5-fluorocytidine) is a new oral fluoropyrimidine carbamate which is converted to 5FU by three enzymatic steps [87], see Figure 1.5.

Capecitabine is initially metabolised in the liver to 5'-deoxy-5-fluorocytidine. It is subsequently metabolised to 5'-deoxy-5-fluorouridine by cytidine deaminase, expressed in higher concentrations in the liver and some solid tumours [88]. The final step is conversion to 5FU by TP an enzyme in higher concentration in tumour cells relative to normal tissues [89]. After oral administration it is extensively absorbed unchanged from the gastrointestinal tract, thereby avoiding the direct release of 5FU from 5'-DFUR by PyNPase in the small intestine. In the clinical

setting its efficacy has been shown to be equivalent to bolus IV 5FU in metastatic colonic carcinoma [90]. Capecitabine achieves long term inhibition of TS and mimics protracted infusional 5FU. It has the additional important advantage of being converted to 5FU predominantly within the tumour cells [91]. There is also evidence that capecitabine (Xeloda) might offer an enhanced therapeutic ratio in chemoradiation schedules.



**Figure 1.5** Conversion of capecitabine to 5FU

1. Xeloda is preferentially activated in tumour tissue by the enzyme thymidine phosphorylase. This selective activation has been validated in humans by a small study in colorectal cancer, which demonstrated 3.2 times the level of 5FU in tumour compared to normal tissues after oral Xeloda [91]. This is in part due to the higher levels of TP found in tumour cells compared to normal tissues [89].
2. In studies performed in several human tumour xenografts, radiation upregulates TP in tumour but not in normal tissue. A single local irradiation of 5Gy increased TP 13 fold at 9 days after irradiation [92].

3. In the WiDr colon and MX-1 mammary human tumour xenograft models, the combination of a single local X-ray irradiation with either Xeloda or 5'-dFUrd was much more effective than either radiation or chemotherapy alone. In contrast, treatment with radiotherapy and 5FU in combination showed at most additive effects [92].
4. Sawada [93] has also shown that TP expression can also be induced by other chemotherapy agents including MMC and the taxanes, the same induction of TP has also recently been demonstrated for cyclophosphamide [94].

For this reason Xeloda is now being investigated as an alternative to IV 5FU in many chemotherapy regimens for solid tumours. This includes several phase II trials in both anal and rectal carcinomas.

## **1.21 Chemoradiation in anorectal carcinomas**

Chemoradiation is being increasingly used in the treatment of many cancer sites. In the case of anal cancer it has changed current practice in the last decade. In rectal cancer its role is still to be clearly defined and we await current trials. It appears to be most promising in downstaging locally advanced rectal cancers to ensure, where possible, a curative resection with negative margins. Most chemoradiation schedules deliver a dose of 45-50.4Gy in 25-28 fractions over 5-6 weeks with synchronous chemotherapy delivered in days 1-5 and 29-33. The origin of this schedule lies in the work of Charles Moertel published in 1969 [95] and Norman Nigro in 1974 [14]. This introductory chapter explains in detail the current role of chemoradiation in the treatment of anal and rectal carcinomas.

## **Section 4            The role of molecular markers**

### **1.22            Introduction**

Prognostic markers are relevant factors in the management of patients with cancer. They help to stratify patients for treatment by identifying different risk groups in order to improve survival and reduce morbidity [96]. These markers include biological, clinical, pathological and genetic features. Currently clinical and pathological features are used commonly in treatment decision-making for patients with cancer. In anal and rectal cancers the extent of the primary tumour (T stage) and lymphatic spread (N stage), as well as certain histological features are already established prognostic indicators. However, these factors are not easy to predict preoperatively. Thus there have been considerable efforts to define accurate histological, pathological and molecular information preoperatively, which could be used to predict outcome.

In the future it may become possible for clinicians to individualise the treatment of patients with cancer by tailoring the treatment based on the molecular biology of each individual's tumour. By understanding the tumour biology, molecular markers could be identified, which may be predictive of both patient response and outcome to treatment, and are independent from the traditional clinical and pathologic factors. Although the combination of radiotherapy and chemotherapy holds great promise for the treatment of both anal and locally advanced rectal cancer, survival rates could be much improved. In developing potential prognostic and predictive markers in these two disease sites it could help the clinician identify those patients most at risk of local recurrence and metastatic disease and treat them with more intensive chemoradiation regimens or in the case of anal cancer, primary surgery.

Many of these molecular marker proteins can be assayed using standard immunohistochemical techniques, from formalin-fixed, paraffin-embedded tissue or by western blot or flow cytometric analysis. Other laboratory techniques have been developed to identify specific genetic alterations using the reverse-transcriptase polymerase chain reaction and DNA sequencing. However, achieving a reliable and informative predictive marker is not a trivial task due to the complexity, redundancy and interdependence of the biological processes that determine how a tumour will

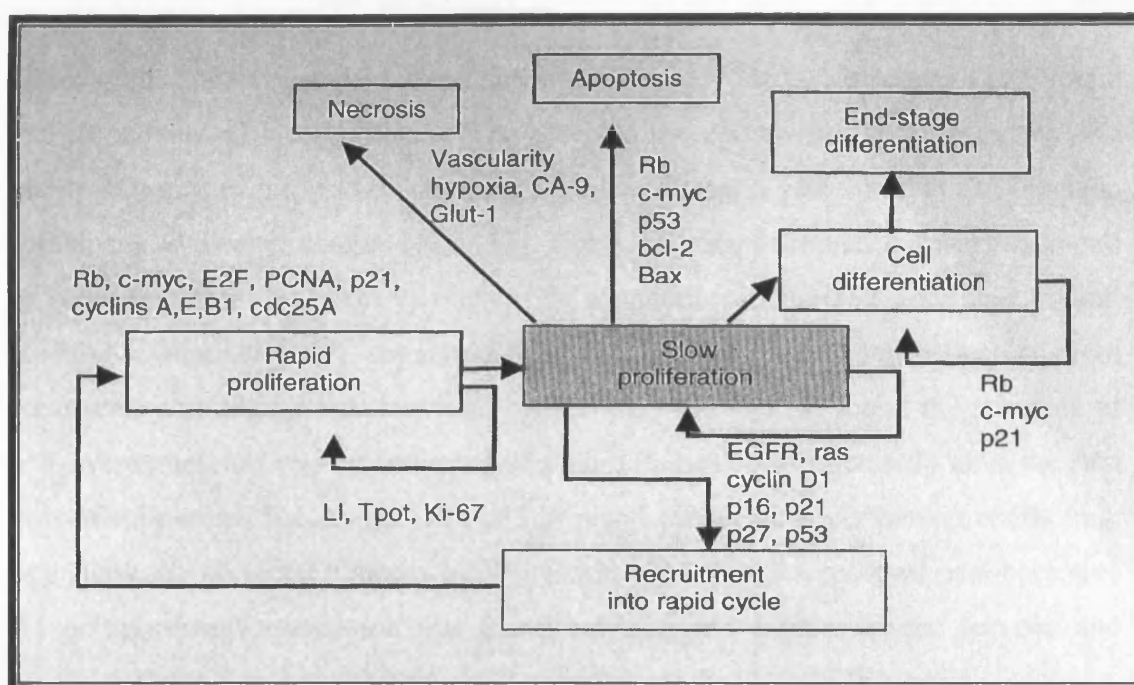


respond to the radiotherapy, chemotherapy or a combination of the two treatment modalities.

### 1.23 Choice of molecular markers

There are potentially numerous molecular markers, which could be studied in predicting the response to combined chemotherapy and radiotherapy, and survival. The two treatment modalities may interact in many ways; they may be active against different tumour cell subpopulations and chemotherapy may inhibit the repair of sublethal damage of tumour cells after radiation exposure.

One approach is to consider key elements in each of the major pathways in the context of tumour structure, see Figure 1.6 [97] and proteins involved in 5FU metabolism. This can provide a basic profile of tumours, which can then be augmented with further parameters. This approach has determined the choice of markers for this study. All of the proteins analysed have been shown in previous studies to be potential candidates for predicting both prognosis and response to 5FU-based chemoradiation.



**Figure 1.6** The complex pathways of tumour growth [97]

### 1.23.1 P53

The human p53 gene encompasses 20kb of DNA on the short arm of chromosome 17 [98]. It is a tumour suppressor gene which plays a critical role in cell-cycle control and apoptosis [99]. In response to DNA damage p53 is upregulated leading to G1 cell cycle arrest, allowing DNA repair to occur, thus limiting propagation of the genetic abnormalities. Mutations in this gene are believed to be the most common genetic abnormality in human tumours providing cells with a selective growth advantage [100]. Many clinical studies have suggested that a mutated p53 gene is a marker for adverse prognosis [101] and poor response to treatment in various tumour types [99, 102, 103]. A very close correlation between over-expression of p53 protein, on immunohistochemistry, and mutations of the p53 gene has been described [104, 105].

In rectal cancer non-functional p53 can be found in up to 84% of cases [106]. In a study by Spitz [107] p53 overexpression correlated inversely with pathological response to pre-operative chemoradiation in 45 locally advanced rectal tumour specimens. Similar associations between p53 expression and a poor response to radiotherapy/chemotherapy were found in studies by Qiu [108] and Luna-Pérez [109].

Other studies however have not found this association [110]. Rebischung [111] found that in patients with rectal cancer who received pre-operative RT, the presence of a p53 mutation was associated with a significantly shorter 5 year survival and this is in agreement with other studies [112-114]. Goh [115] demonstrated a lower likelihood of benefiting from adjuvant therapy when a mutation in the p53 gene was present [116]. Schwandner [117] suggested that p53 had an independent effect on both recurrence and disease-free survival. Interestingly Diez [118] found the presence of p53 overexpression was an indicator of a high risk of recurrence only after the first year of follow-up. The results with p53 in rectal cancer however remain conflicting. In a study of 146 rectal tumours by Wiggensraad [119] which received post-operative RT no significant association was found between p53 expression and survival and several other studies have also reported negative results [119-122].

In anal carcinomas overexpression of p53 has been demonstrated [123-125]. In a study by Bonin of 64 anal carcinomas [126] there was a trend for patients whose

tumours overexpressed p53 to have inferior loco-regional control and absolute survival, however this was not statistically significant. Tanum's study [124] of 113 patients also did not show the expression of p53 to be of prognostic significance. However, Wong demonstrated in [125] patients with anal carcinoma that p53 expression was an independent prognostic marker for disease-free survival and in a study by Mullerat [127] looking at pre-invasive precursor lesions, p53 expression increased significantly as the lesions became more dysplastic and invasive. This has led to the hypothesis that p53 is involved in the progression of anal cancer. Alterations in p53 protein function in anal cancers may result from sequestration by the E6 viral oncoprotein of the human papilloma virus (HPV) [123, 128, 129].

### **1.23.2 Ki-67**

Ki-67 is one of the most commonly used markers of proliferation. It equates to a measure of growth fraction and shows considerable heterogeneity between individual tumours. It is expressed within all phases of the cell cycle except G0. In rectal cancer fast proliferating tumours have been shown to demonstrate a high immediate response to high dose radiation therapy [130] and an improved response to chemoradiation [131, 132]. In a study by Adell [133] using short-course preoperative radiotherapy a high Ki-67 predicted for an increased risk of treatment failure suggesting that those patients whose tumours demonstrated a higher proliferation rate should be offered the longer course chemoradiation schedules.

The apoptotic index is a marker of cell loss which is related to cell proliferation. It may therefore be a surrogate marker for proliferation. In rectal cancer, a reduced local recurrence is observed following both surgery alone and preoperative chemoradiation, in tumours demonstrating a higher apoptotic index [134, 135]. In cancers with a lower apoptotic index, recurrence rates were improved with the addition of pre-operative radiotherapy [134].

Evidence to date has revealed that anal carcinomas have a high proliferation rate with a high incidence of aneuploidy [136]; however the prognostic significance of this is uncertain. In the study by Mullerat [127] Ki-67 expression, in addition to p53, significantly increased with progression from dysplastic lesions to invasive anal carcinoma. It may therefore be a useful marker of early dysplasia. This finding was

also found in a study by Calore [137] of low grade intra-epithelial neoplasia in HIV, where Ki-67 expression correlated with recurrence. Similar studies have not shown a prognostic correlation with Ki-67 in anal cancer [138, 139].

### **1.23.3 Bcl-2**

Bcl-2 is a membrane bound protein found predominantly in the nuclear envelope, endoplasmic reticulum and mitochondria [140]. Bcl-2 was discovered in a B-cell lymphoma in which a 14:18 translocation placed bcl-2 under control of the promoter for the immunoglobulin heavy chain gene, a rearrangement leading to abnormal expression of the bcl-2 protein [141]. Bcl-2 expression is common in many tissues in the embryo but is uncommon in adult tissues with a few exceptions such as B cells and intestinal crypt cells. However, a wide variety of malignancies do express bcl-2 including 60% of follicular lymphomas and commonly breast cancer [142, 143]. Bcl-2 is a negative regulator of apoptosis and when active it inhibits the induction of apoptosis from a wide variety of stimuli including radiation and chemotherapy [144]. Overexpression potentially promotes resistance to both treatment modalities.

In rectal carcinoma Kim [132] found a higher rate of complete pathological response to pre-operative chemoradiotherapy in bcl-2 negative tumours, however other studies have failed to confirm this [108, 110, 132, 135]. In studies by Buglioni [116] and Schwandner [117] bcl-2 expression was associated with an improved prognosis in colorectal carcinomas. However, Bhatavdekar [145] demonstrated that in Dukes C colorectal carcinoma patients there was a significant poorer overall survival if the tumours were bcl-2 positive.

In anal carcinoma there is currently little published data on the prognostic role of bcl-2. A recent study by Allal et al [146] demonstrated that a lack of bcl-2 expression correlated with a lower local control and 5-year survival, and this remained significant on multivariate analysis.

### **1.23.4 Cyclins**

The cyclins are a family of regulatory proteins, which interact with cyclin-dependent kinases (CDK's). These are responsible for regulation of cell cycle transitions. Cyclin A is a marker of proliferation which is expressed predominantly by cells in the S and

G2 phases of the cell cycle. It is useful in conjunction with ki67. Cyclin D1 binds cdk4, and this complex promotes cell cycle progression by phosphorylation of the retinoblastoma protein, which is required for transition through the G1/S checkpoint. Overexpression of cyclin D1 could facilitate the process of repopulation by recruitment and it is found commonly overexpressed in head and neck cancers [147]. Several studies in the same tumour site have suggested that cyclin D1 expression may provide independent prognostic value for clinical outcome [148, 149].

Cyclins D1 and E have been found to be over-expressed in colorectal carcinoma [150], however in a study by Schwandner [151] cyclin D1 was not found to be associated with prognosis in rectal cancer. There is little further data on the potential prognostic role of cyclins in anorectal carcinomas.

#### **1.23.5            Carbonic Anhydrase 9**

Tumours which are low in oxygen are often associated with a poor response to radiotherapy [152] and as a result are less curable. Markers which can identify the amount of hypoxia within a tumour are therefore potentially important.

CA9 is a member of the carbonic anhydrase family which catalyses the reversible hydration of carbon dioxide to carbonic acid. Transcription of this gene is known to be regulated by the Von Hippel-Lindau tumour suppressor gene, the protein product of which is part of a ubiquitin ligase complex [153]. This complex is responsible for targeting HIF-1 $\alpha$  for oxygen-dependent proteolysis [154, 155]. The interaction of Von Hippel-Lindau protein with HIF-1 $\alpha$  appears to be governed by iron-dependent hydroxylation of a specific proline in the oxygen-dependent degradation domain of HIF-1 $\alpha$  [156, 157]. Therefore at low levels of oxygen, HIF-1 $\alpha$  is stabilised causing an increase in expression of CA9. Areas of high expression of CA9 have been shown to co-localise with regions of tumour hypoxia in bladder, skin and more recently cervix cancer, and incubation of tumour cells under hypoxia has been shown to induce expression of CA9 [158-161].

Expression of CA9 occurs in several tumour sites [158]. It was recently found to be a significant predictor of disease-specific and metastasis-free survival in patients with squamous cell cancer of the cervix [162]. The role of hypoxia in rectal and anal

carcinomas and how it may influence response to chemoradiation is largely unexplored with little or no published data. Hypoxia in other tumour sites especially squamous cell carcinomas has been shown to be important in predicting response to treatment and influencing clinical outcome. Determining the effect of hypoxia in squamous anal carcinomas was therefore of particular interest.

#### **1.23.6 CD34**

There is considerable interest in angiogenesis and its role in the growth of solid tumours. Measuring micro-vessel density (MVD) within tumours has been suggested as an indirect way of studying angiogenesis, which may act as a powerful prognostic tool. CD34 stains small and large vessels with equal intensity in normal and tumour tissues.

Some investigators have shown a relationship between high MVD and lymph node and distant metastases in colorectal cancer [163-165]. Galindo-Gallego [166] showed that the presence of microvessels was prognostic for overall survival but not progression free survival, at all Dukes staging. This was supported by Takebayashi [165]. In a study by Vermeulen [163] 145 colorectal tumours were examined. A high MVD was associated with a shorter survival in those patients undergoing surgery. This group also showed that a high MVD correlated with a higher incidence of haematogenous but not lymphogenic metastases, suggesting that in those patients with a Dukes B tumour with a high MVD, adjuvant chemotherapy should be considered. Interestingly a study by Saclarides [167] demonstrated an inverse association between a high concentration MVD and decreased survival, this was also found in the study by Qui [108] following pre-operative RT and surgery. These conflicting results may arise because they involve selection of areas near or at the edge of a tumour to identify the highest areas of activity, thereby introducing selection bias.

In anal carcinoma there is little data on the potential role of angiogenic markers. In a study of CD31 [168], a platelet endothelial cell adhesion molecule, like CD34, no correlation with neoplastic relapse was noted but there was a significant correlation with the depth of tumour invasion. This finding supports the concept that tumour growth is angiogenesis-dependent.

### **1.23.7 Thymidylate synthase (TS)**

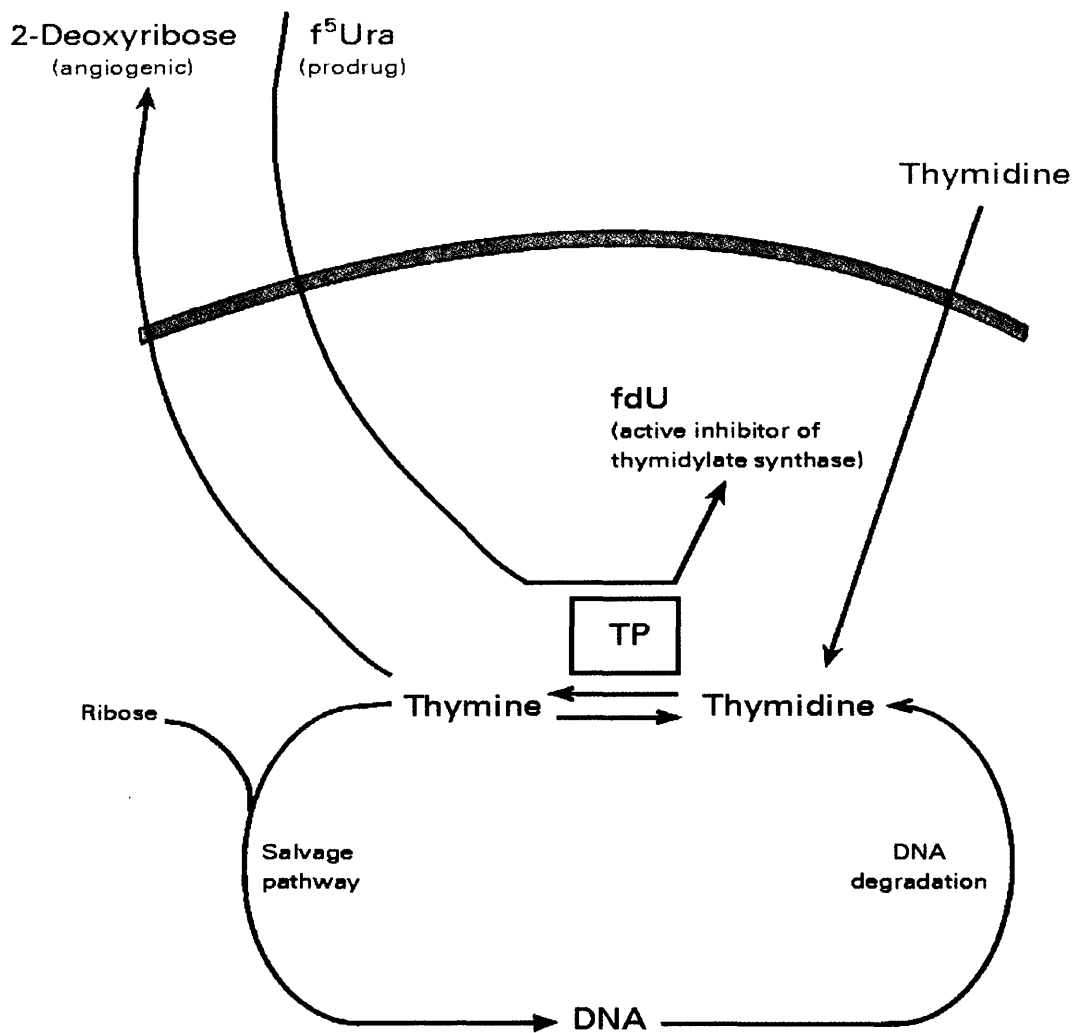
Thymidylate synthase (TS) is the enzyme required for the production of thymidine in DNA synthesis, and its inhibition is one of the primary mechanisms by which 5-fluorouracil (5FU) inhibits growth of colorectal carcinoma. TS is therefore a rate-limiting enzyme in the DNA synthetic pathway. TS levels within a tumour are associated with 5FU sensitivity and overexpression of TS is associated with an impaired response to anti-metabolite treatment and thus a poorer survival [169].

High levels of TS have been shown to correlate with high rates of recurrence and decreased survival in colorectal cancer patients particularly in those treated with 5FU [170, 171]. In a study [171] of patients entered into the NSABP-R01, 49% of patients with low TS levels were free of disease at 5 years compared to 27% in those with high TS levels. In Edler's study [172] high TS protein expression predicted for a poorer disease-free survival and overall survival independently of Dukes stage and 5FU treatment. Okonkwo [110] demonstrated that the TS level in rectal tumours could be used as a predictor of sensitivity to chemoradiation and in a study by Saw [173] of 60 locally advanced rectal carcinomas treated with pre-operative chemoradiation the presence of no TS staining was associated with more significant downstaging.

Although there is a large body of evidence for the role of TS as a prognostic and predictive marker in colorectal cancer, its role in anal cancer remains largely unexplored.

### **1.23.8 Thymidine phosphorylase**

TP is an enzyme, which is involved in the synthesis of DNA and catalyses the reversible synthesis of thymidine. See Figure 1.7. It is also identical to platelet-derived endothelial cell growth factor, a marker of angiogenesis. Takebayashi [165] found that a surrogate marker for microvessel counts was the expression of thymidine phosphorylase and this group demonstrated that TP expression was significantly associated with a worse outcome in 163 primary colorectal carcinomas. This has been shown in other studies [170, 174]. Metzger [175] found that a high level of expression of TP was associated with a poorer response to 5FU, in a small study of



**Figure 1.7** Physiological role of thymidine phosphorylase



colorectal cancers. There is little data however looking at the role of TP in rectal cancer alone and in anal cancer.

#### **1.23.9 Dihydropyrimidine dehydrogenase (DPD)**

DPD is the initial and rate-limiting enzyme in 5FU catabolism [176]. It is active in a circadian pattern [76, 176]. This is associated with an inverse circadian pattern in fluorouracil concentrations [76, 177]. There is considerable inter-patient variability in DPD activity in normal tissues, primarily the liver. This variability has a normal distribution with up to a sixfold variation from the lowest to the highest levels [76, 176, 177]. Malignant tissues can also vary in their level of DPD activity [178].

The importance of DPD has been demonstrated in several studies. Low levels of DPD are associated with higher response rates to 5FU chemotherapy in several tumour types [75, 179, 180] resulting in an improved prognosis. It is also an indicator of 5FU-related toxicity.

#### **1.23.10 Epidermal growth factor receptor**

EGFR is a 170-kD trans-membrane glycoprotein whose gene is located on the short arm of chromosome 7p12. It is one of four members of the erbB family of receptors that has an intracellular domain possessing intrinsic tyrosine kinase activity. On binding with its ligands, EGF or transforming growth factor alpha (TGF- $\alpha$ ), EGFR initiates transduction signals regulating cell growth and proliferation [181, 182]. EGFR is overexpressed in a wide variety of tumour types and its overexpression has been associated with more aggressive tumour behaviour, adverse patient survival and poor tumour response to conventional therapy [183-186].

A recent paper [187] examined tumour specimens of patients with rectal cancer treated by preoperative chemoradiation. 64% demonstrated EGFR + tumours. EGFR positivity was not associated with either the primary stage or the nodal stage of the tumour. However it was significantly associated with a poorer response to chemoradiation and a poorer DFS. If this could be validated, EGFR expression could identify patients at high risk of failure who might benefit from novel therapeutic modalities, which target this receptor.

### **1.24 The role of molecular markers in anal carcinoma**

It can be seen from the information already presented that anal carcinoma has been the subject of little study in terms of molecular markers. Those studies which have been undertaken have found no convincing correlation between prognostic marker expression and response to therapy or clinical outcome. This is primarily because of the small number of patients studied.

Therefore, there is clearly a need to expand the biological knowledge of anal carcinoma and to investigate relevant markers related to treatment and outcome.

### **1.25 The role of molecular markers in rectal carcinoma**

Locally advanced rectal cancer offers a unique model for defining histological and genetic changes because access to histological material is relatively easy without the requirements of an anaesthetic. Rectal cancers develop along a variety of molecular pathways. Each tumour involves multiple, sequential genetic alterations different from the next. Many markers have been studied, however the specific prognostic and predictive value of these in rectal cancer has yet to be definitively validated. This is in part due to the use of polyclonal and monoclonal antibodies, different scoring systems and stage of disease and differing treatments. The majority of studies are also small and have combined colon with rectal cancers and so the effect of these markers in rectal cancer, which is often biologically more aggressive, is unclear.

### **1.26 In conclusion**

An evidence-based approach to identifying the most valuable prognostic markers for anal and rectal cancer is clearly important. A frequent difficulty in assessing the clinical value of these markers is the relatively small number of patients in each study. What is needed is the study of tumours in large prospective randomised trials using similar staining and scoring systems so that results can be compared between institutions. At present, based on the evidence to date it is too early to incorporate these predictive markers into the routine diagnostic workup to give information to the clinician and help with decisions regarding treatment in these two disease sites.

The purpose of this study was to expand further the knowledge of the potential use of these molecular markers and in particular with relevance to response to 5FU-based chemoradiation treatment. In this study a large number of anal and rectal cancer specimens have been analysed and the anal specimens were from patients who had been randomised into a large phase III randomised trial.

## **Chapter 2:**

### **Methods and Materials**

## **Chapter 2                      Methods and Materials**

### **2.1                      Immunohistochemistry (IHC)**

#### **2.1.1                      Handling of specimens**

Formalin fixed, paraffin embedded blocks of rectal and anal cancer specimens were received. Samples were requested for all patients with squamous anal carcinoma entered into the ACT I trial and 155 patients with locally advanced carcinoma of the rectum who had received pre-operative 5FU based chemoradiation. A total of 240 anal and 60 rectal cancer tissue blocks were received. Samples were cut into 4µm sections using a rotary microtome. Sections were then mounted onto poly-L-Lysine coated microscope slides and dried overnight at 37°C.

#### **2.1.2                      Primary antibodies**

The choice of molecular markers used for the study of the rectal and anal cancer specimens is explained in Chapter 4 of the introduction.

##### **2.1.2.1                      P53**

IHC staining of p53 was achieved using a mouse monoclonal antibody against human p53 protein (DAKO p53, DO-7 clone. Isotype IgG2b) code number M 7001. This is a recombinant human wild type protein expressed in E.coli. The DO-7 clone recognises an epitope in the N-terminus of the human p53 protein. This epitope is known to reside between amino acids 19 to 26 and will therefore react with both mutant and wild type protein. P53 immunohistochemical staining has been widely used in the study of p53 status in multiple cancers. The wild-type p53 protein has a short half-life and is virtually undetectable by immunohistochemical staining. However, mutations in the p53 gene often result in stabilisation of the protein, and accumulation of p53 protein has been demonstrated to correlate with p53 mutations [105]. P53 staining is nuclear.

##### **2.1.2.2                      Ki-67**

Staining was achieved using a mouse monoclonal antibody (DAKO Ki-67, clone MIB-1. Isotype IgG1) code number M 7240. This is a recombinant human peptide corresponding to a 1002 bp Ki-67 cDNA fragment. The Ki-67 antigen is a nuclear

protein, which is defined by its reactivity with monoclonal antibody from the Ki-67 clone. It reacts with an epitope encoded by a 66 bp repetitive element in the Ki-67 gene. During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes.

#### **2.1.2.3 Bcl-2**

A mouse monoclonal antibody was used (DAKO bcl-2, clone 124. Isotype IgG1) code number M 0887. This is a synthetic peptide comprising amino acids 41-54 of human bcl-2 protein. The protein is associated with mitochondria, smooth endoplasmic reticulum and the perinuclear membrane and plays a central role in the inhibition of apoptosis (programmed cell death). Staining is therefore cytoplasmic and perinuclear.

#### **2.1.2.4 Cyclin A**

Staining for cyclin A was achieved using a mouse monoclonal antibody (Novocastra cyclin A, clone 6E6. Isotype IgG1), code NCL-Cyclin A. This is a recombinant human protein corresponding to the N-terminal fragment of the cyclin A protein. Cyclin A is a proliferation marker which is detectable in S phase into G2. Expression of cyclin A is within the nucleus.

#### **2.1.2.5 Cyclin D1**

An antibody derived from a prokaryotic recombinant fusion protein corresponding to the human cyclin D1 molecule was used for staining (Novocastra cyclin D1, clone P2D11F11. Isotype IgG2a). Cyclin D1 is a 36kD protein, which is normally detectable in the G1 phase of the cell cycle. Staining is predominantly nuclear; however cytoplasmic staining may also be seen, especially in formalin-fixed tissues and when overexpression is present.

#### **2.1.2.6 CD34**

Vasculature staining was achieved using a mouse monoclonal antibody against human endothelial cells (DAKO CD34, clone QBEnd 10 – class II. Isotype IgG1) code number M7165. The CD34 antigen is a single chain transmembrane protein of approximately 116,000 Mr, expressed on immature haematopoietic stem/progenitor cells and small-vessel endothelial cells [188]. It appears to be expressed at its highest

level on the earliest progenitors, and to decrease progressively with maturation [189]. It stains small and large vessels with equal intensity in normal and tumour tissues. However it may also stain some lymphatic and perivascular stroma [190].

#### **2.1.2.7 CA9**

Staining for hypoxia was assessed using a CA9 antibody, provided by Professor Adrian Harris, Oxford Radcliffe Hospital. This was a mouse monoclonal anti-human antibody M75 [161]. CA9 is strongly induced by hypoxia in a broad range of cell types [161] and is usually associated with areas of necrosis [191]. Membrane and cytoplasmic staining occurs.

#### **2.1.2.8 Thymidine Phosphorylase**

TP, a marker of angiogenesis, was stained for using a mouse monoclonal antibody (Neomarkers thymidine phosphorylase, clone P-GF.44C. Isotype IgG1) code number #MS-499-P. This is a recombinant full-length human thymidine phosphorylase. TP is both chemotactic and mitogenic for endothelial cells and a non-heparin binding angiogenic factor present in platelets. Its enzymatic activity is crucial for angiogenic activity. Its staining occurs in the nucleus, cytoplasm and stroma.

#### **2.1.2.9 Thymidylate Synthase**

TS is a marker of DNA synthesis and also plays a critical role in the effect of 5FU chemotherapy. This protein was stained using a polyclonal antibody supplied by Dr Wynne Aherne, Institute of Cancer Research, Sutton. It was a rabbit anti-human antibody [192, 193] and staining occurred predominantly within the cytoplasm.

#### **2.1.2.10 Dihydropyrimidine Dehydrogenase**

DPD is the enzyme responsible for the catabolism of 5FU within the liver to an inactive form. DPD was stained using an antibody supplied by Dr Masakazu Fukushima, Hanno Research Centre, Saitama, Japan. This is a rabbit anti-recombinant human DPD polyclonal antibody (IgG) [194]. DPD activity is present exclusively in the cytosol and its expression is highest in liver tissue, but it is also expressed in variant levels in other normal and cancer tissue.

### **2.1.2.11 Epidermal growth factor receptor**

Staining for EGFR was assessed using an antibody specific for the external domain of the epidermal growth factor receptor. A recombinant fusion protein corresponding to the human EGFR molecule was used (Novocastra EGFR, clone 113. Isotype IgG2a), code NCL-L-EGFR. Its role is in the regulation of cell division, proliferation and differentiation [181, 182, 195]. The pattern of staining varies between tumour types. In squamous carcinomas the predominant pattern is membrane staining but in adenocarcinomas cytoplasmic staining is more common.

## **2.1.3 Detection systems**

### **2.1.3.1 Avidin-Biotin Complex method**

All stains except CA9 and EGFR were performed using the Avidin-Biotin Complex method, see Figure 2.1. This method depends on the high affinity of avidin or streptavidin for biotin through four binding sites. A biotinylated immunoglobulin acts as a link antibody, comprising of biotin molecules covalently bound to the constant regions of the heavy chain. Open sites on a separate avidin-biotin complex bind to the biotin on the link antibody. The avidin-biotin complex also consists of horseradish peroxidase, which oxidises the chromogen, diaminobenzine tetrahydrochloride (DAB).

Visualisation of the antigen/antibody reactions is dependent on the ability to produce an insoluble coloured product at the site of the reaction. Peroxidase-conjugated antibodies achieve this when in contact with an electron donor and in the presence of hydrogen peroxide. DAB is one of a number of electron donor molecules used as chromogens in peroxidase-based immunohistochemistry. The staining procedure involves adding DAB and hydrogen peroxide to the avidin-biotin complex; the peroxidase releases atomic oxygen from the hydrogen peroxide that oxidises DAB to produce a brown insoluble end product. Prior to this, the endogenous cellular peroxidases need to be blocked to ensure the stain is specific to bound antibody. This is achieved by bathing samples in 0.1% hydrogen peroxide before adding the antibody.



### **2.1.3.2 Envision method**

This staining method was used for CA9 and EGFR (DAKO Envision-HRP mouse) code K4006. This system uses unique chain-polymer based technology, in which the peroxidase-labelled polymer is conjugated with secondary antibodies against rabbit or mouse. The system is biotin-free, resulting in minimal background staining, and consists of only two antibody stages, thus saving time. Figure 2.2 illustrates this system.

### **2.1.4 Control sections**

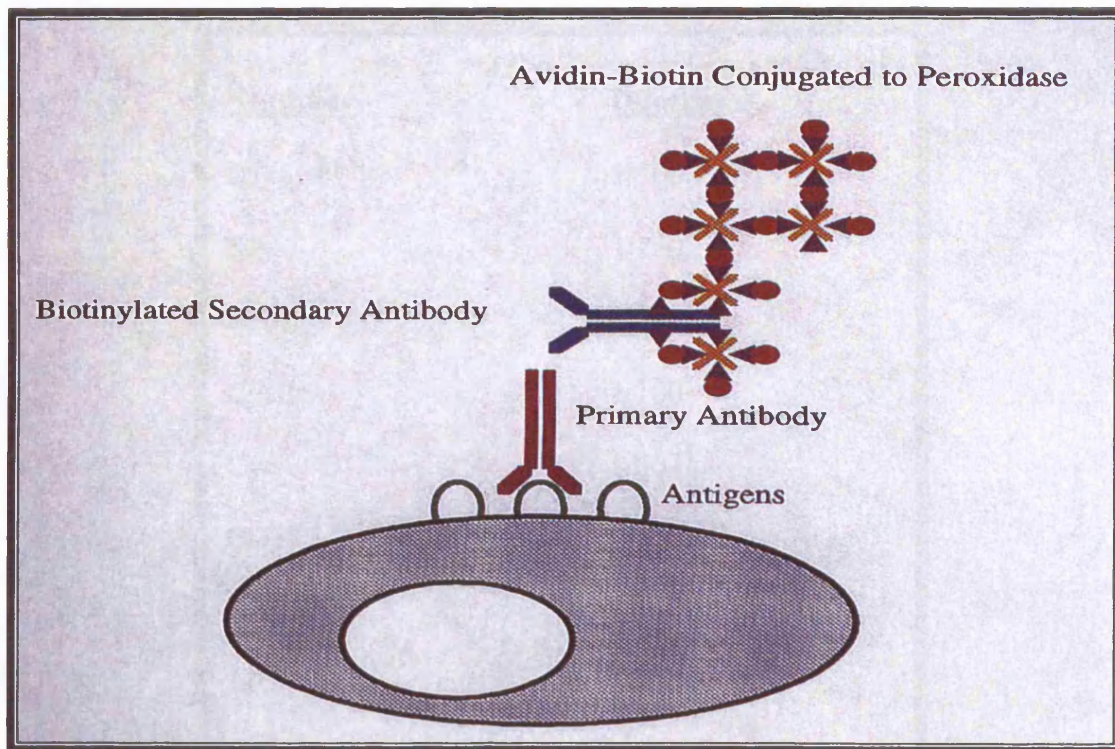
In order to ensure that each immunohistochemical run had been correctly performed a positive control sample was stained concurrently for each particular antibody. These were;

- Tonsil for the proliferative and endothelial cell markers – CD34, bcl-2, Ki-67, cyclin A
- Mantle cell lymphoma for cyclin D1
- Bladder squamous cell carcinoma for CA9 and EGFR
- Colon adenocarcinoma for DPD and TS
- Breast adenocarcinoma for p53 and TP

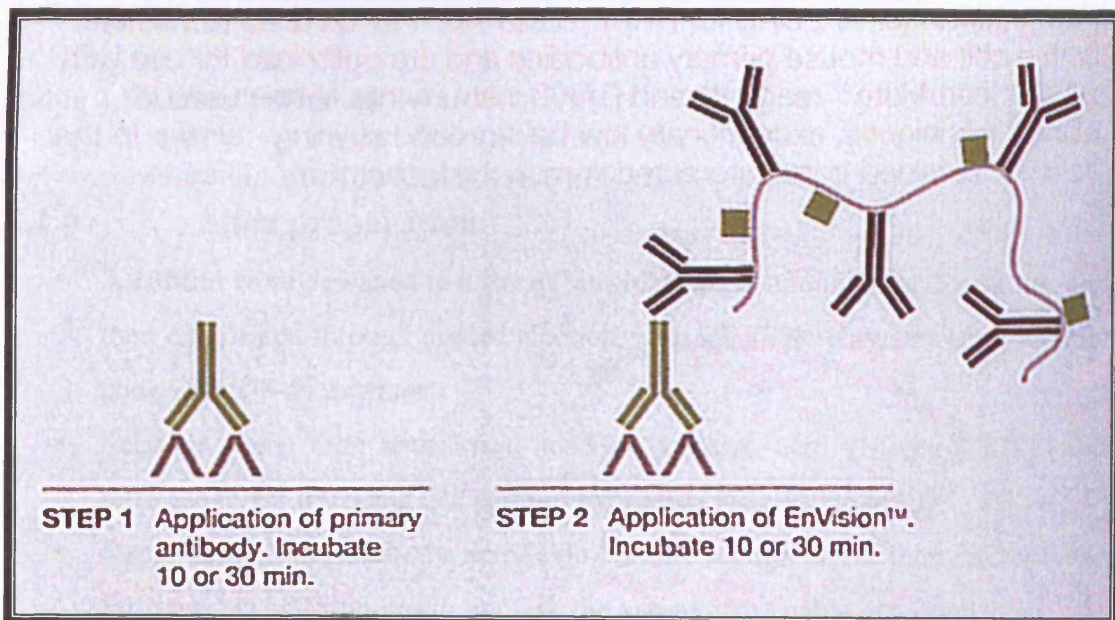
### **2.1.5 Preparation of antibodies for staining**

All antibodies were diluted to their appropriate concentrations in antibody diluent (DAKO chemMate) code S2022.

The dilutions used for each antibody are given below in Table 2.1.



**Figure 2.1** Avidin-Biotin Complex method [196]



**Figure 2.2** Envision method [196]

<u>Antibody</u>	<u>Dilution</u>
P53	1/300
Ki-67	1/250
Bcl-2	1/300
Cyclin A	1/100
Cyclin D1	1/60
CD34	1/200
CA9	1/50
TP	1/400
TS	1/300
DPD	1/500
EGFR	1/20

**Table 2.1** Antibody dilutions

### 2.1.6 Slide preparation

- All slides were dewaxed in xylene (Surgipath code 03665E) for 5 minutes and then rehydrated through graded alcohols 100, 90, 70% (Hayman ethyl alcohol code 200-578-6) to water
- Sections were then transferred to 10mM citric acid (Merck 277814 N) buffered to pH 6.0 using 2M sodium hydroxide (BDH102524X)
- Depending on the antibody, see Table 2.2, the sections were then microwaved for intervals of 4 minutes to unmask the antigens to enable detection
- Slides were then left to cool for 10-20 minutes depending on the antigen being stained and then washed in running tap water for a further 5 minutes
- Staining was then performed using an automated system or manually using the Envision method

<u>Microwave times</u>	<u>Antibody</u>
3 x 4 minutes	P53 Ki-67 Bcl-2 Cyc A Cyc D DPD CD34
4 x 4 minutes	TS EGFR
No pre-treatment microwave	TP CA9

**Table 2.2** Pre-treatment microwave times

## 2.1.7 Staining Methods

### 2.1.7.1 Automated procedure for staining

An automated system, the Dako Autostainer (universal staining system model LV-1), see Figure 2.3, was used for staining all of the antibodies except CA9 and EGFR. The automated system enabled an increased output with improved reproducibility and quality. The system is open and involves the slides being secured in a horizontal position, see Figure 2.4. The reagents are then dispensed onto the tissue by use of teflon-coated pipettes, thus mimicking manual staining. The streptavidin-biotin complex method was used in this automated system.

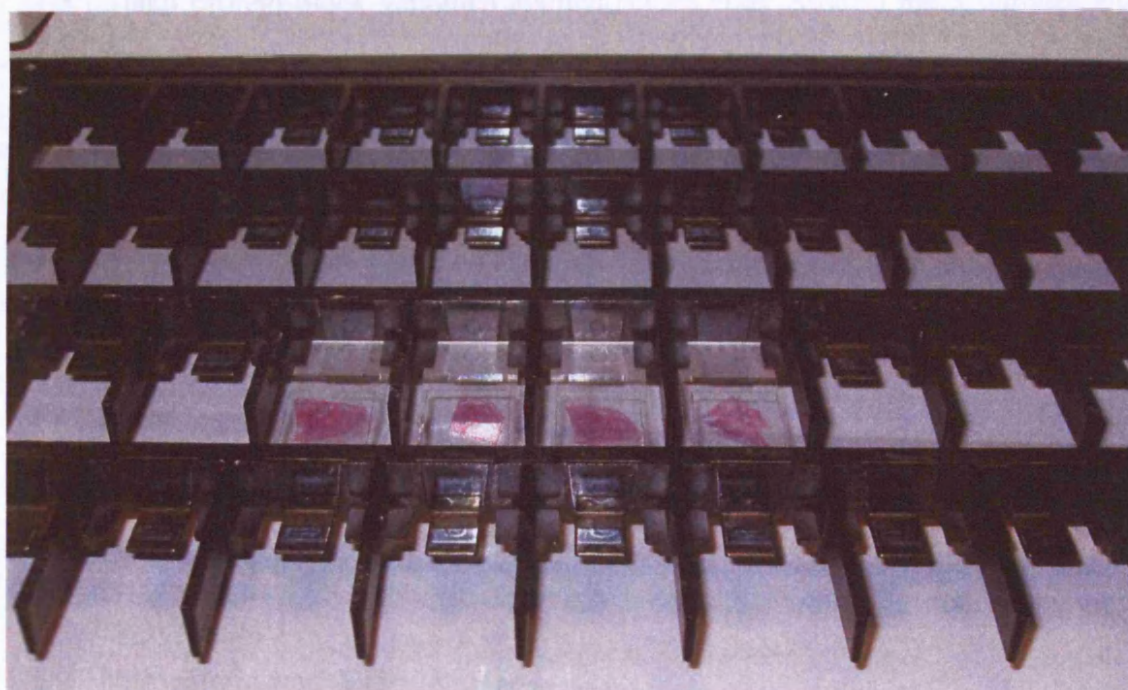
### Procedure

- Endogenous peroxidase was blocked using Dako chemMate peroxidase block, code S2023 for 5 minutes
- Slides were then washed well in distilled water and then in Tris buffered saline (TBS) with 0.0004% tween-20 (TBS + T)
- The slides were incubated for 60 minutes with each primary antibody





**Figure 2.3** Dako Autostainer



**Figure 2.4** Positioning of slides in automated system

- Slides were washed in TBS before applying the biotinylated secondary antibodies (AB2, ChemMate detection kit - Dako, code K5001) for 30 minutes
- After washing in TBS + T the Streptavidin peroxidase (HRP) was added (ChemMate detection kit – Dako K5001) for 30 minutes
- Following further washes in TBS + T, DAB substrate (ChemMate detection kit- Dako K5001) was added for five minutes following which further washes with TBS-T took place
- The slides were then removed from the autostainer and washed well in running water

#### **2.1.7.2 Envision procedure for staining for CA9**

- Endogenous peroxidase was blocked using Dako peroxidase block (Envision kit code K4006) for 5 minutes
- Slides were then washed well in running tap water
- Dako protein block was then applied (Dako code X0909) for 5 minutes and excess protein block was tipped off
- CA9 antibody was then applied for 30 minutes
- Slides were then washed in TBS three times before applying Dako envision HRP mouse polymer (code K4006) for 30 minutes
- Slides were then washed again three times in TBS
- DAB substrate (Envision kit code K4006) was then applied for 5 minutes
- Slides were then rinsed in TBS and rinsed well in running tap water

### **2.1.8 Solutions**

#### **2.1.8.1 Tris buffer**

0.1M Tris (BDH103156)

0.1M Hydrochloric acid (BDH28507)

Adjust to pH 7.4-7.6

#### **2.1.8.2 Tris buffer saline (TBS)**

500ml Tris buffer

4500ml Distilled water

40.5 grams Sodium chloride (Sigma S-7653)

Adjust pH to 7.4-7.6

#### **2.1.8.3 Tris buffer saline with tween (TBS+T)**

5 litres Tris buffer saline pH 7.4-7.6

2mls Tween (Tween 20- DAKO code S1966)

#### **2.1.9 Mounting slides**

All slides were then counterstained with Gills haematoxylin (Surgipath Gill 1, code 01500E) for 5-10 seconds and washed well in running tap water. They were then dehydrated through graded alcohols and xylene before mounting the coverslips with p-Xylene-bis-N-pyridinium bromide (DPX) (Surgipath code number 08600E).

#### **2.1.10 Image analysis**

A Zeiss Axioscope trans-illumination microscope with 5x, 10x, 20x and 40x objectives and a 10x eyepiece connected to a 3 CCD colour camera (JVC KY55F 1/3" 6.4 x 4.8mm) was used to visualise and capture tissue section images. Images were digitised with a Matrox Meteor<sup>TM</sup> frame grabber in a PCI bus 600MHz Pentium<sup>TM</sup> desktop PC. Image capture procedures were standardised for light intensity and background subtraction at different magnifications, depending on the analysis required, using routines developed in Visilog 5.02 software (Noesis Vision Inc.).

#### **2.1.11 Scoring of antibody expression**

All antibodies except for CD34 were scored for both intensity and percentage of tumour cells stained. Intensity was scored by assessing the variation across the specimens for each marker and a semi-quantitative scoring system was constructed as either negative (score 0), weak (score 1), moderate (score 2) or strong (score 3). The percentage of cells stained was scored in increments, the limits of which varied with each marker, see Table 2.3. In the case of TP, staining could occur in the stroma, cytoplasm or nucleus, for this reason an intensity and percentage score was given for each location.

CD34 staining was scored as the average number of vessels per  $\text{mm}^2$ . This involved counting the number of vessels on 10 random high power fields (x10 eyepiece and x40 objective) and an average calculated from the scores. From the known field size at this power the number of vessels per  $\text{mm}^2$  could be calculated. For the purpose of the analysis the scores were then subdivided into quartiles.

Three investigators who were blind to the clinical outcome evaluated all the marker scores independently. The full scoring system for each marker is given in Table 2.3



<b>Markers</b>	
<b>Field</b>	<b>Coding</b>
<b>Grade</b>	1= Well differentiated 2= Moderate 3= Poor
<b>Intensity score</b> (used for all markers except CD34)	0= None 1= Mild 2= Moderate 3= Strong
<b>Bcl2 % of cells stained</b>	0= None 1= <20% 2= 20-50% 3= >50%
<b>P53 % of cells stained</b>	0= 0-5% 1= 5-20% 2= >20-50% 3= >50-75% 4= >75%
<b>Ki67 pattern</b>	1= Marginal 2= Random 3= Mixed
<b>Ki67 % of cells stained</b>	0= None 1= <5% 2= 5-20% 3= >20-40% 4= >40%
<b>TS % of cells stained</b>	0=<5% 1=5-20% 2=>20-50% 3=>50-75% 4=>75%
<b>TP % of cells stained</b> <b>Nucleus</b> <b>Cytoplasm</b> <b>Stroma</b>	0= None 1= <20% 2= 20-50% 3= >50%

<b>DPD % of cells stained</b>	0= 0-5% 1= >5-20% 2= >20-50% 3= >50%																
<b>CAIX % of cells stained</b>	0= None 1= <5% 2= 5-20% 3= >20%																
<b>CD34 score</b>	10 fields at x10 objective and x40 power Average number of vessels per mm <sup>2</sup> <table><tr><td></td><td><u>Median</u></td><td><u>Range</u></td></tr><tr><td><b>First quartile</b></td><td>48.30</td><td>28-&lt;60</td></tr><tr><td><b>Second quartile</b></td><td>67.85</td><td>60-&lt;76</td></tr><tr><td><b>Third quartile</b></td><td>85.10</td><td>76-&lt;94</td></tr><tr><td><b>Fourth quartile</b></td><td>105.80</td><td>94-233</td></tr></table>			<u>Median</u>	<u>Range</u>	<b>First quartile</b>	48.30	28-<60	<b>Second quartile</b>	67.85	60-<76	<b>Third quartile</b>	85.10	76-<94	<b>Fourth quartile</b>	105.80	94-233
	<u>Median</u>	<u>Range</u>															
<b>First quartile</b>	48.30	28-<60															
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<b>Third quartile</b>	85.10	76-<94															
<b>Fourth quartile</b>	105.80	94-233															
<b>Cyclin A % of cells stained</b>	0= 0-5% 1= >5-10% 2= >10-20% 3= >20-50%																
<b>Cyclin D presence of staining</b>	0= None 1= Positive nuclear 2= positive cytoplasm																
<b>EGFR % of cells stained</b>	0=<5% 1=5-20% 2=>20-50% 3=>50-75% 4=>75%																

**Table 2.3** Scoring of molecular markers

## **2.2 Cell lines and culture conditions**

### **2.2.1 Cell culture medium**

HT29 and SW48 human colon cancer cells obtained from the European Collection of Animal Cell Cultures, were routinely grown and maintained as monolayers in tissue culture flasks. All tissue culture was performed under sterile conditions, using EMEMS (Eagle's Minimal Essential Medium – Sigma M4655) supplemented with 10% Foetal Calf Serum (Helena Biosciences), 1x non-essential amino acids (Sigma M7145), 1mM sodium pyruvate (Sigma S8636), 7.5% sodium bicarbonate (Sigma S8761), 200mM glutamine (Sigma G7513) and penicillin-streptomycin solution (Sigma code P4333).

Flasks were maintained in humidified incubators at 37<sup>0</sup>C in an atmosphere of 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>. Cells were collected for treatment or passage by trypsinisation: monolayers were rinsed with sterile phosphate buffered saline (PBS tablets – Sigma P4417) and then incubated briefly with 0.05% trypsin, 0.53mM sodium ethylenediaminetetraacetic acid (EDTA) (Gibco code 15400-054) at 37<sup>0</sup>C. Detached cells were diluted in EMEMS, centrifuged at 1000rpm (revolutions per minute) for 5 minutes and then re-suspended in EMEMS.

### **2.2.2 Mycoplasma testing**

Before commencement of the experiments both cell lines were tested for mycoplasma.

Cells were sub-cultured in antibiotic-free medium in which they were maintained for 3 days prior to testing. They were then trypsinised and approximately  $2 \times 10^2$  cells, to give a confluence of 20-50% at 48-72 hours, in 4mls antibiotic-free medium were inoculated onto a sterile coverslip in a 5cm Petri dish. The cells were then incubated for 72-96 hours. The medium was then poured off and the cells were washed twice with PBS to remove any non-specific DNA and debris. The cell layer was then fixed in 70% industrial methylated spirit (IMS) (Sigma), for 30 minutes and washed with PBS. The cells were then stained with 0.5µg/ml in PBS solution of Hoechst stain (Benzimidazole, Sigma HOE33258) and left at room temperature for 20 minutes under subdued lighting. The stain was then washed off with distilled water and the

cells were inspected using a Nikon UFX-II microscope under UV illumination. The cells were inspected for the presence of bright cytoplasmic staining in the form of discrete dots which represents mycoplasma contamination.

### **2.3 X-irradiation**

Cells from both cell lines were seeded at  $5 \times 10^6$  cells per  $150\text{cm}^2$  flask, in 30mls of EMEMS. They were then irradiated on the fourth day after subculture. The flasks were placed on a perspex baseboard in an incubator maintained at  $37^\circ\text{C}$  and irradiated using a Pantak IV X-ray set. 240 kVp X-rays were delivered at 13 mA (dose rate  $0.5\text{Gy min}^{-1}$ ). In the first set of experiments the cells were exposed to single fractions of 2Gy and 10Gy. In the second set a fractionated course of 2Gy x 4, each fraction delivered 24 hours apart, was given.

### **2.4 Preparation of cell extracts**

For the single fraction doses cell pellets were prepared just after irradiation at 0 hours and then at 2, 7, 24 and 48 hours. The single dose experiments were repeated three times for each cell line so that for each time point, three separate samples were obtained. Similarly in the fractionated experiments three samples were obtained for each 24 hour time point.

Cell pellets were prepared by tipping off the medium from the cells and washing in ice cold PBS. Cells were then scraped from the flasks in 24mls of medium. Half of the sample was fixed for flow cytometry and the remaining was divided into three samples for western blot analysis, thymidine phosphorylase and thymidylate synthase enzyme activity.

### **2.5 Enzyme activity assays**

Once the cell samples had been split for TP and TS analysis, the cells were centrifuged at 1200rpm at  $4^\circ\text{C}$  for five minutes to produce a pellet. Each sample was then washed and centrifuged twice more in 10mls of ice cold PBS to remove as much of the culture medium as possible from the cells. This was to ensure that the Bradford protein assays would be as accurate as possible and not be contaminated by proteins within the EMEMS.

### **2.5.1 Thymidine phosphorylase activity**

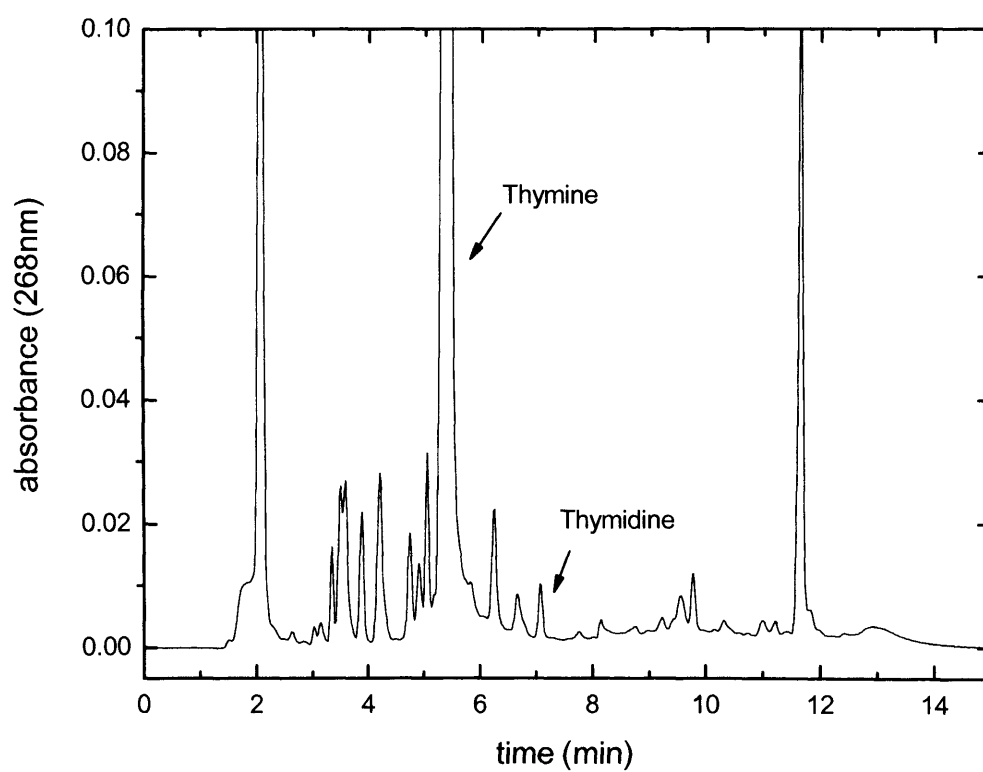
After washing, the excess PBS was tipped off. The cell sample was then harvested on ice after application for 10 minutes of a solution containing 1% Triton x100 (t-Octylphenoxypolyethoxyethanol, Sigma T9284), 50mM Tris-HCL (pH 8.0), 150mM sodium chloride (NACL) (Sigma), 100µg phenyl methyl sulfoxide fluoride ml (Sigma P7626) and 1mg aprotinine/ml (Sigma A1153). Cell suspensions were then centrifuged at 13,000 rpm for 30 minutes at 4<sup>0</sup>C. The supernatants were then stored at -80<sup>0</sup>C until assayed for TP activity. TP enzyme activity was analysed using the method described by Ciccolini [197], which calculates the TP enzyme activity from the amount of thymidine formed in the presence of thymine and a phosphate cofactor.

#### **2.5.1.1 TP enzyme assay**

TP activity was evaluated by incubating 50µl of cell extract in 30mM Tris HCL (pH 7.4), 1mM EDTA, 5mM magnesium chloride (Sigma M2670), and 0.25mM thymine (Sigma T0376). 2.5mM deoxyribose-1- phosphate was added as a cofactor (Sigma D6539). Reactions were stopped after 60 minutes by boiling in a water bath for 5 minutes. The samples were then stored at -20<sup>0</sup>C for 30 minutes. Cell extracts were then centrifuged at 10,000 rpm for 10 minutes. 150µl of supernatant from each of the samples was then loaded onto a Phenomenex Aqua C18 250 x 3.0mm column. Full HPLC conditions are described below. TP activity was expressed as the amount of thymidine formed per mg protein per minute.

#### **2.5.1.2 High performance liquid chromatography analysis**

Detection and separation of the thymidine from thymine was achieved by ion-pair reverse phase chromatography, see Figure 2.5. The HPLC consisted of a Waters 2695 with a 2996 diode array detector. Data was processed using Waters Millennium software. Separation was achieved using a Phenomenex Aqua C18 column at 40<sup>0</sup>C, eluted by (a) 5mM heptane sulphonic acid, 5mM potassium dihydrogen orthophosphate (KH<sub>2</sub>PO<sub>4</sub>), 5mM orthophosphoric acid (H<sub>3</sub>PO<sub>4</sub>) and (b) 75% acetonitrile (all from Fisher Scientific). The separation conditions involved using a gradient of 4-20%b in 9 minutes with a flow rate of 0.6mls/minute. Comparing retention times with standards for thymine and thymidine identified the different peaks.



**Figure 2.5** HPLC separation of thymidine from thymine

## **2.5.2 Thymidylate synthase activity**

After washing, the excess PBS was tipped off. The cell sample was then harvested on ice after resuspending in a solution of 50mM Tris HCL buffer (pH 7.3), containing 2mM dithiothreitol (Sigma D8255). After sonication on an ice bed (three times at 10 second intervals), the cell suspension was immediately centrifuged at 13,000 rpm for 30 minutes at 4°C. The supernatants were then stored at -80°C until assayed for TS activity.

### **2.5.2.1 TS enzyme assay**

TS enzyme activity was measured according to the tritium release assay described by Spears [198]. This assay consisted of incubating 25µl of cytosol with <sup>3</sup>HdUMP (110nM final concentration) (obtained from Amersham Pharmacia Biotech, UK) and 5-10 methylenetetrahydrofolate (mTHF (Sigma) (0.62mM final concentration) in a total volume of 50µl (in the previous Tris-HCL buffer). After 30 minutes of incubation at 37°C the reaction was stopped on ice. The excess of <sup>3</sup>HdUMP was removed by the addition of 300µl of activated charcoal (Sigma) (15%) containing 4% trichloroacetic acid (Sigma) and 5 minutes centrifugation at 10,000 rpm (room temperature). The <sup>3</sup>H<sub>2</sub>O formed during the incubation was then counted in an aliquot of 150µl of the supernatant, to which 10mls of scintillant had been added (Beckman). The scintillation process is described below. Results were expressed as fmoles of <sup>3</sup>H<sub>2</sub>O formed per minute per mg of protein.

The mTHF was prepared from tetrahydrofolic acid according to the method by Moran [199]. This required 50µl of 1M ascorbate (pH6.5), 2.2µl of 37% (vol/vol) formaldehyde and 9.5mls buffer A [1ml of 1M phosphate (pH 7.2), 14µl 2-mercaptoethanol, 2ml of a solution containing 10mg of bovine serum albumin per ml and 17mls of distilled water] to an ampoule containing 3.1µmol of tetrahydrofolic acid (all chemicals from Sigma).

### **2.5.2.2 Scintillation system**

The amount of <sup>3</sup>H<sub>2</sub>O formed was counted using a Beckman LS 6500 Scintillation System. This system features a Motorola 68000 series microprocessor, a digital signal processor and a 32,768 channel Multichannel Analyser. A user programme specific

for  $^3\text{H}$  was used and the instrument calculates counts per minute (CPM). The counts per second can then be calculated and this is equivalent to the amount of radioactivity formed in Becquerels (Bq).

### **2.5.3 Bradford protein assay**

Enzyme activity was calculated as fmoles per minute per mg of protein. The normalisation to the protein content was estimated, in duplicate, using the Bradford protein assay [200]. This uses albumin as a standard. Serial dilutions of both albumin and the enzyme samples were made in a 96 well plate. The Bradford reagent (Sigma) was then added and the plate was analysed on a Labsystems Multiscan MCC/340 MK II. This is an 8 channel vertical light path filter photometer. The absorbance readings were taken at 620nm. The amount of absorption produced is proportional to the protein concentration. A standard curve for protein concentration against absorbance is calculated for albumin. From this curve an estimate can be made of the protein concentration in the enzyme samples.

## **2.6 Protein expression and cell cycle analysis**

In addition to studying the changes in enzyme activity following radiation, the changes in protein expression were also analysed. Flow cytometric analysis was chosen as not only could the relative levels of protein expression be assessed but also the changes in the expression throughout the cell cycle. This is particularly important as cell cycle changes will occur in response to the radiation. The antibodies used for TP (Neomarkers P-GF.44C) and TS (Chemicon International clone TS 106) were mouse monoclonals and in the case of the TP antibody it had not been used previously in flow cytometric analysis. For this reason, prior to commencing the flow analysis, western blots were performed on the earlier samples, in both cell lines to ensure that protein expression was detectable with each antibody.

### **2.6.1 Western blot analysis**

After scraping cells into cold PBS the samples were then centrifuged for 5 minutes at 1200 rpm. The PBS was then tipped off and the pellets resuspended in 1ml of cold PBS and transferred to an eppendorf tube. After further centrifugation at 2000 rpm for 5 minutes the pellets were stored at  $-80^{\circ}\text{C}$  until analysis.



### 2.6.1.1 Western blot

Pellets were resuspended in 150µl of cold lysis buffer (150mM NaCl, 1% Triton X100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 20mM EDTA, distilled water and Tris HCL (pH 7.4) (all Sigma)) and incubated for 1 hour at 4°C. Samples were then sonicated at 100% for one minute prior to resuspending in 150µl of Laemelli Sample buffer (Bio-Rad) containing 10% mercapto-ethanol (Sigma). Samples were then heated at 100°C in a water bath for 3 minutes and then stored on ice.

Samples (20µl) were then loaded into lanes in a stacking gel (4% acrylamide, 0.1% bisacrylamide (both National Diagnostics), 10% SDS, 10% ammonium persulphate (Sigma), 0.1% temed (Sigma) and 0.5M Tris HCL buffer (pH 6.8)) and then subjected to electrophoresis in a separating gel (10% acrylamide, 0.1% bisacrylamide, 0.05% N,N,N',N'-Tetramethylethylenediamine (TEMED), 10% ammonium persulphate, 10% SDS and 1.5M Tris HCL buffer). 20µl of a pre-stained, broad range protein marker (Rainbow marker – Amersham Life Science) was loaded to facilitate identification of the protein bands by molecular weight. Electrophoresis was carried out in a running buffer (10x Tris/Glycine/SDS 0.25mM Tris-1.92M glycine-1% SDS (National Diagnostics)) at 50mA for 4 hours, until the bands were well separated.

Gels were then equilibrated in a pre-chilled transfer buffer (TB) (10x Tris/Glycine 0.2M Tris-1.5M Glycine (National Diagnostics)) at 4°C for ten minutes then positioned on nitrocellulose filters for transfer blotting of protein bands. The cassettes holding the gels and nitrocellulose membrane sandwiched between sheets of filter paper and fibre pads were inserted into an electrophoresis tank filled with TB. Transfer electrophoresis was then applied at 47mA overnight at 4°C.

Once transfer to the nitrocellulose membrane had taken place, the membranes were dried. They were then incubated for 30 minutes in a non-fat milk solution (5% Marvel, 0.2% Tween-20 in PBS) to block non-specific protein binding sites. Each was then incubated for 90 minutes with the appropriate diluted antibody in 2.5% Marvel/PBS-T solution, 1/200 for both TS and TP, and 1/2000 for murine β-actin monoclonal AC-40 (Sigma). The actin was to ensure equal loading of protein. After three washes in 0.2% PBS-T the blots were incubated with secondary goat anti-mouse

IgG antibody conjugated to horseradish peroxidase (DAKO) at 1:1000 dilution in the 2.5% marvel solution, for 45 minutes. The blots were then washed a further three times.

#### **2.6.1.2 Chemiluminescent detection**

An enhanced chemiluminescence (ECL) detection protocol was employed for detection of labelled proteins (Amersham Pharmacia Biotech ECL Kit). The ECL solutions are activated by horseradish peroxidase to emit visible light that can be detected by high performance chemiluminescence film (Hyperfilm ECL, Amersham Pharmacia Biotech). In a dark room, the blots were exposed for 60 seconds to the ECL solutions prior to placing them beneath the hyperfilm. They were then left for 15 minutes and then the film was developed.

### **2.6.2 Flow cytometry**

After scraping the cells in cold PBS the sample was centrifuged at 1200 rpm for 5 minutes. The cell pellet was then resuspended in 0.5mls of cold PBS and syringed gently to break up any clumps. For fixation, 10mls of 70% cold methanol (BDH 101586 B) was added whilst vortexing and the samples were stored at -20<sup>0</sup>C until analysis.

Prior to analysing the experimental samples, titration experiments with both the TS and TP antibodies were performed to determine the optimal concentration of antibody for analysis. This was defined as the dilution at which maximum fluorescence occurred, showing saturation of the antibody. In the case of TP this was at a 1:5 dilution and in TS 1:25.

A further preliminary analysis was carried out to assess the influence of confluency on protein expression of both proteins in each cell line. This was important to determine the appropriate growth conditions of each cell line for the radiation studies.

#### **2.6.2.1 Flow cytometric analysis**

Approximately  $1 \times 10^6$  cells at each time point were placed in two centrifuge tubes; the experiment sample and its control. The cells were then washed twice by

centrifugation at 2000 rpm for 5 minutes in PBS. The remaining PBS was pipetted off and the cell pellets were resuspended with 100 $\mu$ l of the primary antibody or isotypic control for 90 minutes. The antibodies and isotypic control (DAKO) were diluted in 100 $\mu$ l of PBS with 0.5% normal goat serum and 0.5% Tween-20 (PBS-NGS-T (Sigma)). The dilutions are given above. After primary antibody incubation the cells were washed with PBS and incubated for 45 minutes with secondary anti-mouse antibody conjugated to Sigma Fluorescein Isothiocyanate (FITC), in PBS-NGS-T, at a dilution of 1:50. The cells were then washed a further time with PBS prior to resuspending in 0.5mls of a solution of propidium iodide (Sigma) at a concentration of 20 $\mu$ g/ml and ribonuclease at 1mg/ml (Sigma). Samples were then left at 4<sup>0</sup>C for 5 minutes and then analysed.

#### **2.6.2.2 Description of the FACScan**

The flow cytometer system used was a Becton Dickinson's FACScan, which is a laser-based, five-parameter flow cytometry analyser. It consists of a bench-top sensor module and computer system, which controls both acquisition and analysis of data. Cells enter the flow chamber in single file and are irradiated by a 15mW, 488nm air-cooled argon-ion laser. Dichroic mirrors spectrally filter emitted light, separating and deflecting longer wavelengths whilst transmitting shorter wavelengths. Scattered laser light is collected in both the forward (narrow angle) and side direction. The longer wavelengths are further separated by a series of long pass and bend pass filters and directed to three photomultiplier detectors, FL1, FL2, and FL3. Having reached the photomultiplier the signal is amplified, digitised, processed and stored on the computer module. The FL1 detector measures light in the green range of the spectrum (515 to 545nm) and is optimised for FITC detection. FL2 measures orange-red light (564-606nm) emitted by phycoerythrin whilst the FL3 detector transmits wavelengths in excess of 650nm, suitable for detection of red light emitted by propidium iodide, which binds to DNA.

#### **2.6.2.3 Data analysis**

Data was analysed using a computer acquisition/analysis programme, Cellquest (Becton Dickinson, San Jose, Calif). Events were quantified by the imposition of computer generated windows (CGW) to define specific regions on the histogram or two-dimensional dot plot. CGW allows the definition of specific populations of

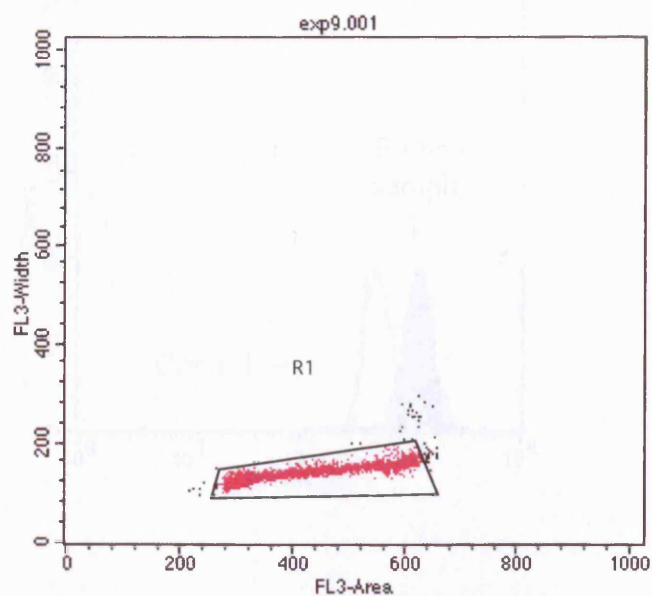
nuclei or cells and required phases of the cell cycle. Regions can be set around populations of cells to omit extraneous interference from debris or from populations of cells whose data is not required. In this analysis, the doublet discrimination capability of the FACScan was used to exclude debris and cell doublets from further analysis. This was achieved by creating a dot plot of FL3 area versus FL3 width, see Figure 2.6. A region was placed around the single cell population as shown and this was used to gate the data. Analysis was carried out on a gated dot plot of DNA content (FL3-area) against FITC (FL1-height) for each protein, see Figures 2.7 and 2.8.

#### **2.6.2.4 Calculation of TS and TP protein levels**

Using Cellquest, TS and TP positivity was calculated from the comparison of the number of events within regions applied to the dot plots of both the control and test sample. Initially a region is set around the control dot plot of FL3 area v FL1 height. This region demarcates labelled from unlabelled nuclei. This same region is superimposed on the antibody-labelled dot plot of the same specimen and the event count is subtracted from that of the control. This gives the overall number of nuclei showing specific labelling due to TS or TP protein expression. Further regions can be set around populations of nuclei, which lie within different phases of the cell-cycle, to allow analysis of oncoprotein expression within each phase of the cell cycle. The median fluorescence of the FITC-labelled nuclei gives an estimate of the relative amount of protein contained within each cell cycle phase and this can be represented numerically as a ratio of the median fluorescence of the positive cells compared to the isotypic control (unlabelled) cells.

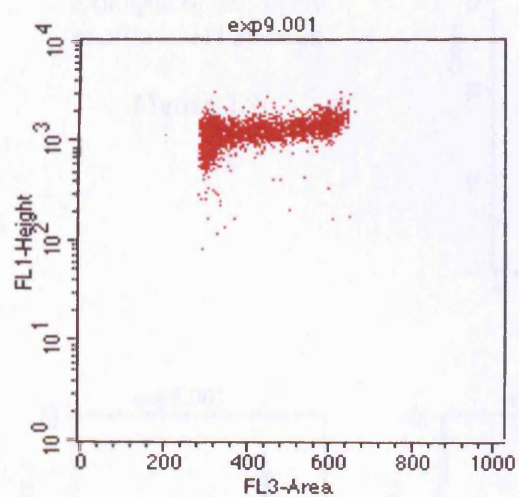
For the purpose of this study the computer system was programmed to collect 5000 cell events and generate a dot plot of FL3-area v FL3-width for each test sample and control. A region was then set around those nuclei adhering specifically to the FITC secondary antibody. This was done by excluding the small proportion of nuclei staining non-specifically and the labelled cell debris exhibiting the highest fluorescence values, which is estimated to be less than 2% of the whole population of labelled material. The median fluorescence of the test and control was then calculated for each. A histogram of FL3 area v counts was then generated and regions were then set on each phase of the cell cycle, see Figure 2.9, this histogram gives the cell cycle

profile of the labelled nuclei, see Figure 2.10. The number of events and median fluorescence were then calculated for each phase of the cell cycle and compared between test and control samples.

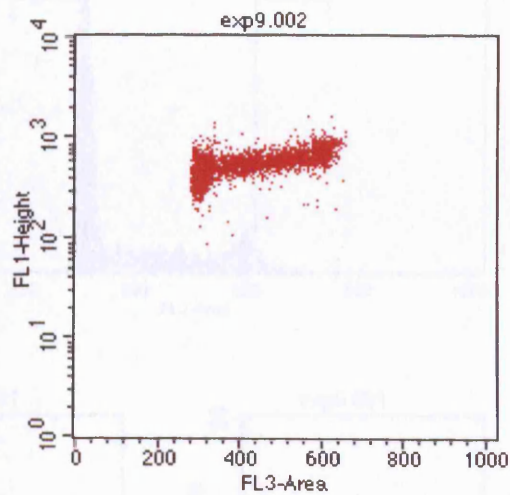


**Region 1 set around nuclei  
with specific binding to FITC**

**Figure 2.6** FL3-area vs. FL3-width

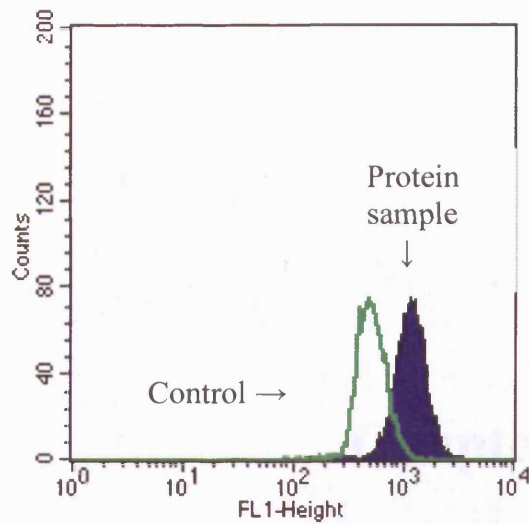


**Protein Sample**



**Isotypic control**

**Figure 2.7** FL3-Area vs. FL1-Height after gating

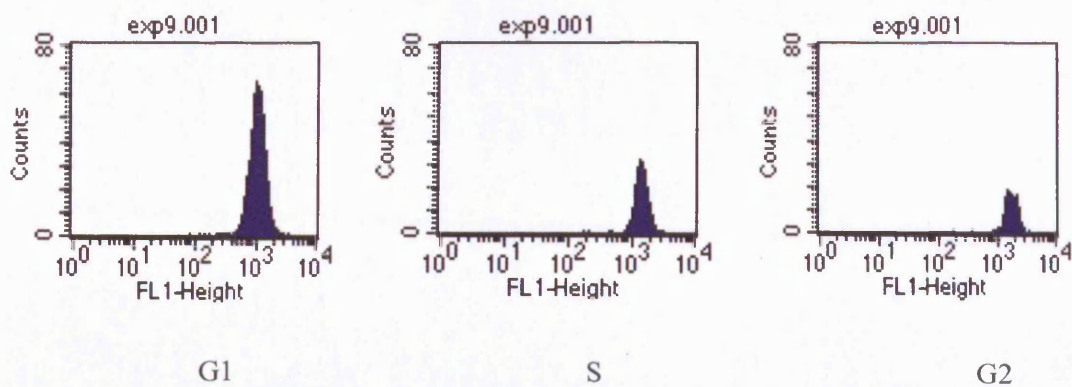
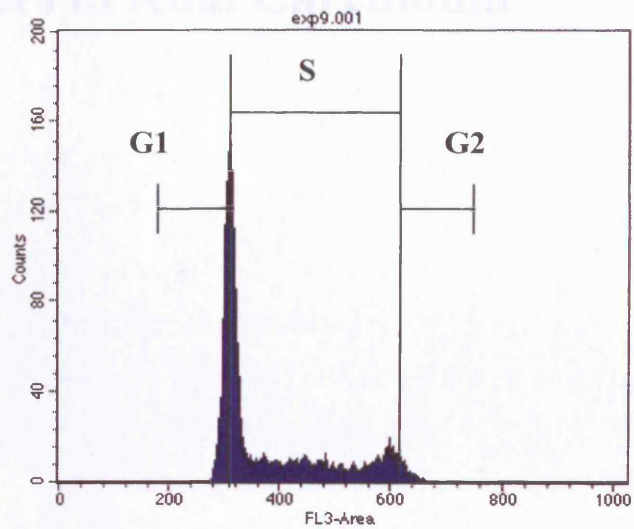


Median fluorescence of  
the protein sample vs  
isotypic control

**Figure 2.8**

Example of cell cycle  
profile in HT29 cells

**Figure 2.9**



**Figure 2.10** Cell cycle profile for labelled nuclei

## **Chapter 3:**

# **Molecular Markers in Anal Carcinoma**



## Chapter 3

### Defining potential predictive and prognostic molecular markers in anal carcinoma

#### 3.1 Aims

- Define predictive molecular markers for the effectiveness of chemoradiation
- Develop a panel of biological and clinical parameters, that will predict the outcome of sub-groups of patients within each arm of the trial
- Identify new prognostic indices that will predict the outcome to treatments, indicating the best choice of treatment or the need to find an alternative treatment in anal carcinoma

#### 3.2 Introduction

The ACT I trial was one of the largest randomised phase III trials undertaken in anal carcinoma, recruiting a total of 585 patients with squamous cell carcinoma of the anus. The patients were randomised to receive radiotherapy alone or in combination with 5FU and MMC, see Introduction for trial algorithm. Permission from the CRC trials office (Cancer Research Campaign) was given to collect and study histological material obtained from the trial.

A panel of 10 molecular markers were examined. These markers were chosen as indices of proliferation, vascularity and cell cycle control. The contribution of thymidylate synthase (TS), thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase were also studied in the context of the 5FU chemotherapy.

Proliferation:	Ki-67 and cyclin A
Recruitment:	Cyclin D1 and p53
Redistribution:	Cyclin A
Apoptosis:	P53 and bcl-2
Hypoxia:	CA9
Tumour structure:	Grade

Vascularity: CD34  
5FU: TS, TP and DPD

The panel of molecular markers were examined to provide a basic profile of the tumours. This profile was analysed in conjunction with known prognostic pathological and clinical indices, which were recorded as part of the trial database. These included sex, age, site of disease; canal or margin, tumour stage, grade, treatment arm and tumour response to treatment. The markers were also augmented with drug and radiation specific parameters.

### **3.2.1 Marker Staining**

Figure 3.1 demonstrates the staining patterns for each marker in the anal carcinoma specimens. The patterns of staining for each marker were discussed in the methods and materials chapter.

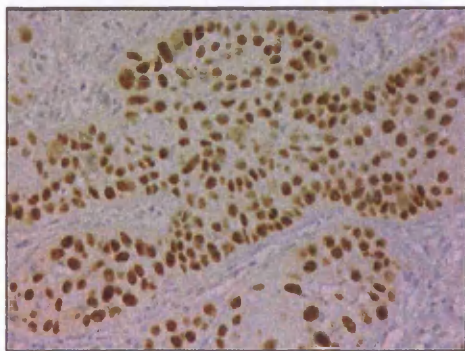
## **3.3 Handling of specimens**

Formalin fixed, paraffin embedded blocks from the original diagnostic biopsies were requested from the relevant pathology centres. Samples were requested for all patients entered into the ACT I trial. A total of 240 tissue blocks were received. Samples were cut into 4µm sections using a rotary microtome. Sections were then mounted onto poly-L-Lysine coated microscope slides and dried overnight at 37°C.

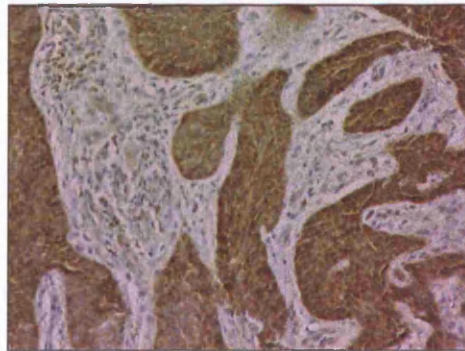
## **3.4 Statistical analysis**

The main endpoint was relapse-free survival (RFS), with secondary endpoints of cause-specific survival (CSS) and response to chemoradiation.

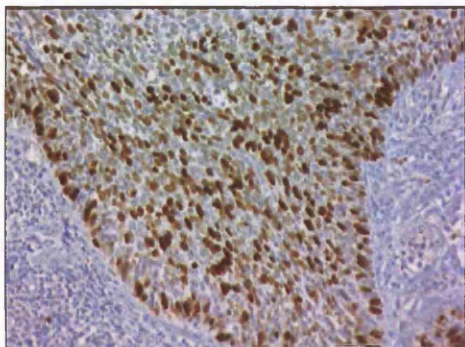
A univariate analysis was performed using Kaplan-Meier curves for the association between each of the markers and known pathological prognostic indices for RFS, CSS and overall survival (OS). Comparison between the different marker scores with the endpoints was compared by the logrank test and associations between markers were assessed using the Spearmans Rank Correlation Coefficient.



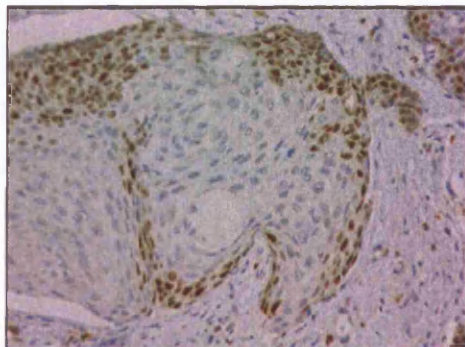
P53



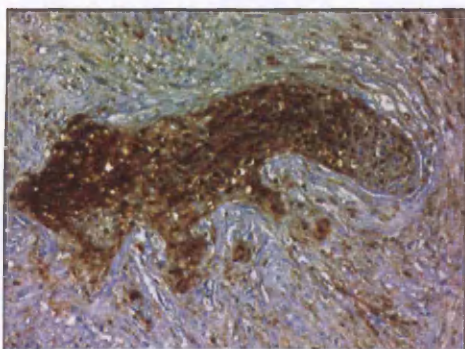
Bcl-2



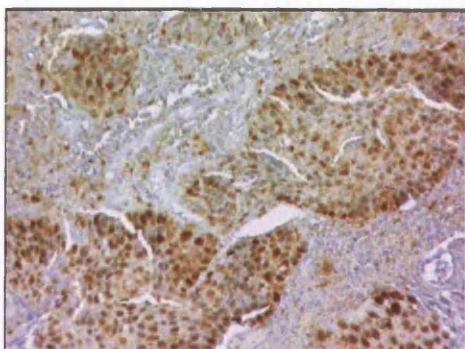
Ki67



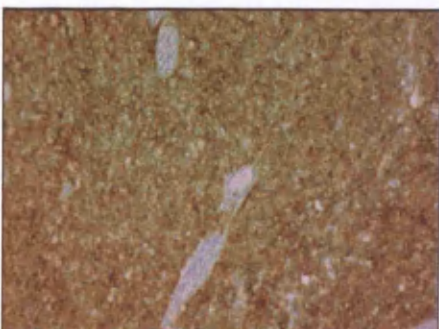
Ki-67 marginal pattern



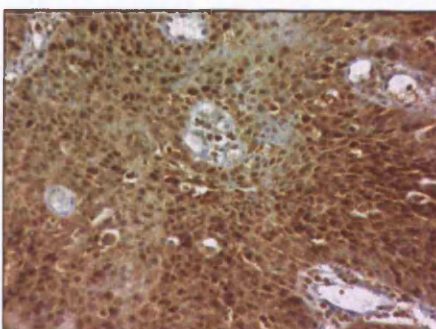
TP mixed



TP nuclear

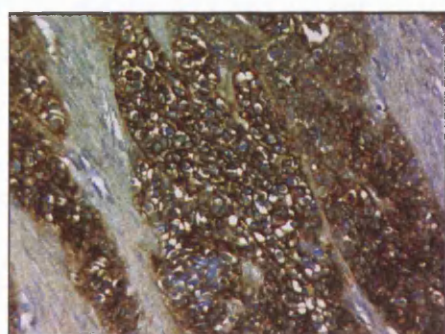


TP cytoplasmic

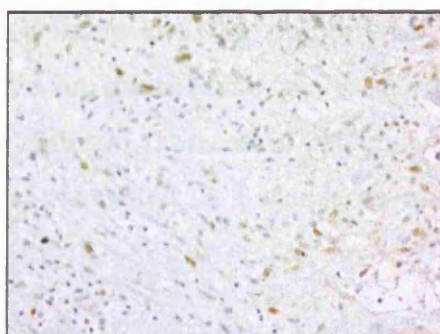


TS

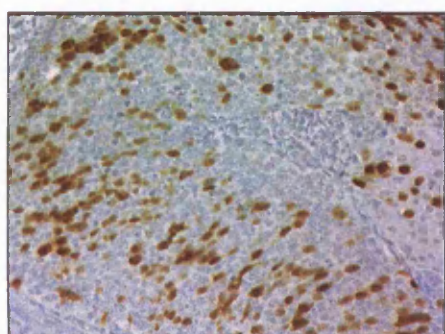




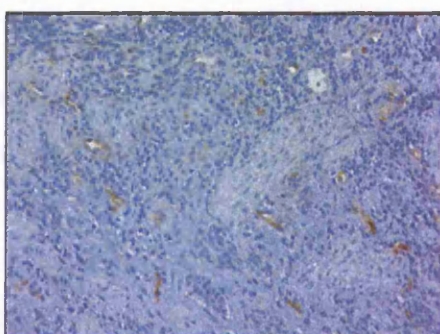
CA9



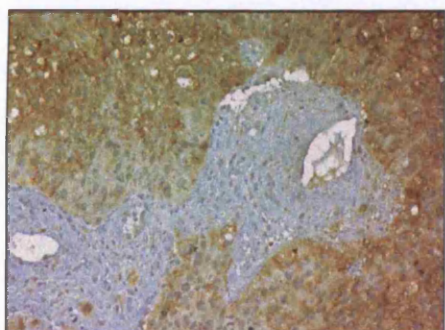
Cyclin D1



Cyclin A



CD34



DPD

**Figure 3.1** Examples of molecular marker expression patterns in anal cancer

A multivariate analysis was then undertaken using the Cox proportional hazards model. Those indices, which were positive on multivariate analysis, were then compared between the two treatment arms. Hazard ratios were calculated and an estimate of benefit from chemoradiation was calculated ( $\pm 1$  standard error of the estimate).

For the analysis the statistics package used was SPSS for windows release 11.5.0 (Statistical package for Social Sciences).

### 3.4.1 Cox proportional hazards model (PHM)

This is a multiple regression analysis of survival data [201]. The basic assumption in the PHM is that at time (t) the hazard rate (death rate) for a patient with prognostic factors or covariates  $z_1, z_2, \dots, z_p$  is given by

$$\lambda(t; z_1, \dots, z_p) = \lambda_0(t) \cdot \exp(\beta_1 z_1 + \dots + \beta_p z_p)$$

where  $\lambda_0(t)$  is an unspecified baseline hazard function, and  $\beta_1, \beta_2, \dots, \beta_p$  are the regression variables to be estimated from the analysis.

The sign of a regression variable ( $\beta_i$ ) indicates whether an increase in the corresponding covariate ( $z_i$ ) results in an improved ( $\beta_i$  negative) or a worsened ( $\beta_i$  positive) prognosis. If  $\beta_i$  is not significantly different from zero, the  $i$ 'th covariate has no significant influence on prognosis.

## 3.5 RESULTS

### 3.5.1 Patient characteristics

Of the 240 blocks received there was missing data on 10 patients; therefore these were excluded from the analysis. The median age was 63 years (range 29-88) with a male:female ratio 99:131 (43%:57%). The predominant site was anal canal and palpable nodes were present in 16%. The majority of anal carcinomas (211/230) had a complete or  $>50\%$  response to treatment. The median follow-up was 42 months and in this time 100 patients died. The majority of these deaths (76%) were related to their anal carcinoma. Patient and tumour characteristics within each treatment group, and comparisons with the original ACT I trial data, are shown in Table 3.1. This table demonstrates that the clinical characteristics of the cohort of patients obtained for this

study, were comparable with the characteristics of the complete ACT I trial dataset. In the ACT I trial, tumour grade was not documented and the addition of chemotherapy did not impact upon treatment compliance.

### **3.5.2 Distribution of molecular markers in the study**

The following sections describe the distribution of staining positivity and staining intensity for each of the groups of markers investigated in the study. Correlation analysis was used to assess the significance of any associations between intensity and positivity.

The distribution of marker scores between the individual treatment groups is shown and demonstrates that there is an equal distribution of scores for each marker between the two treatment arms.

#### **3.5.2.1 Proliferation markers: Ki-67 and cyclin A**

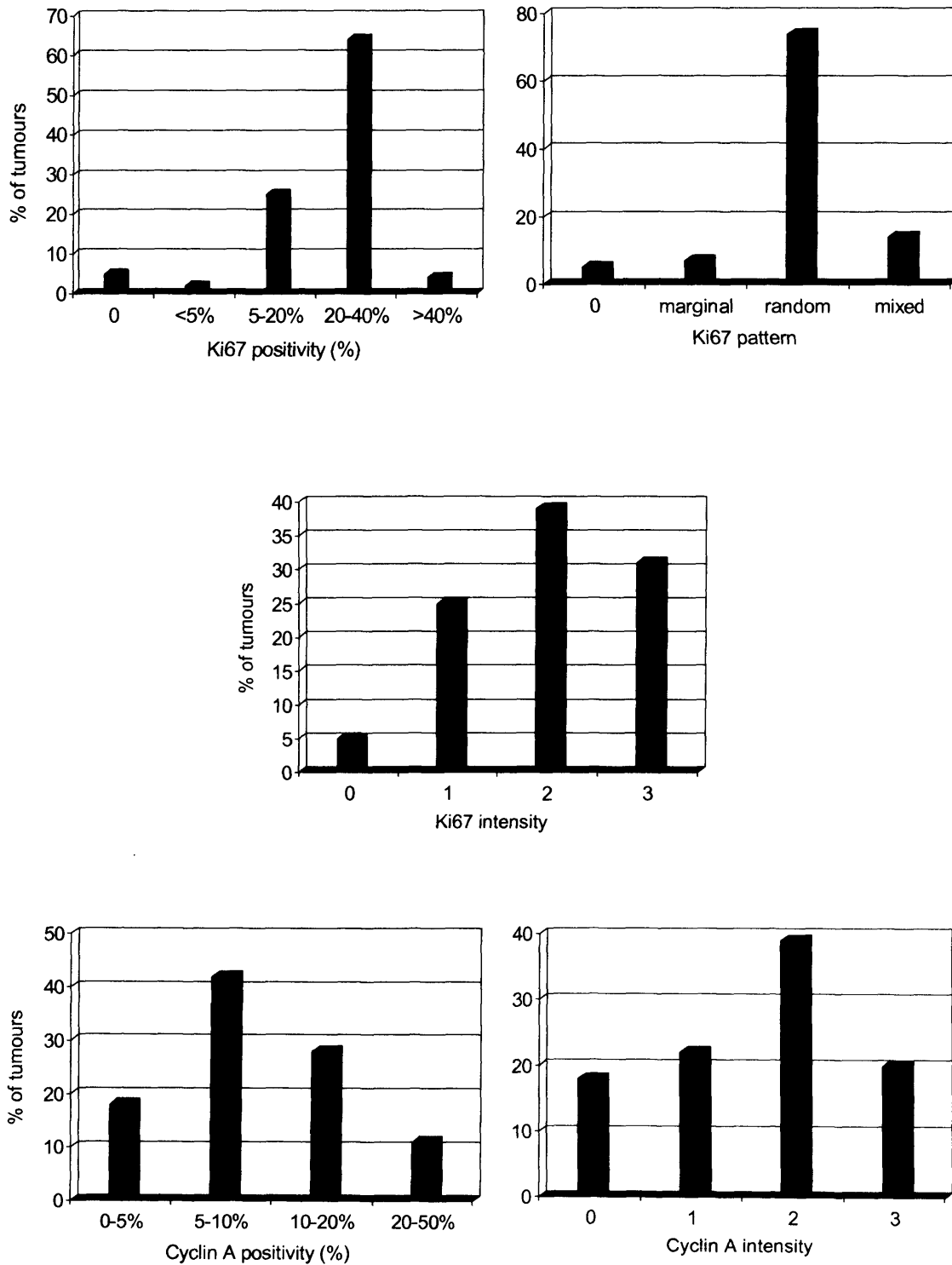
Fig. 3. 2 shows that Ki-67 expression was detectable in nearly all patients (94%) as was cyclin A. The majority of tumours had Ki-67 indices that exceeded 20%. In most patients (>70%) the distribution of proliferation was random. The percentage of cells staining correlated significantly with both Ki67 pattern ( $r^2 = 0.239$ ,  $p < 0.001$ ) and intensity ( $r^2 = 0.522$ ,  $p < 0.001$ ). Cyclin A staining was less than 20% in about 90% of the tumours in agreement with its expression pattern confined to S and G2 cells. Greater positivity for cyclin A correlated with the intensity of staining ( $r^2 = 0.719$ ,  $p < 0.001$ ).

#### **3.5.2.2 Apoptosis markers: bcl-2 and p53**

The anti-apoptotic protein, bcl-2, was detected in 38% of the anal tumours with variable levels of staining intensity (Fig. 3.3). Of the tumours that were positive, the majority showed positivity in over 50% of the tumour cells. There was a strong correlation between positivity and intensity of staining ( $r^2 = 0.966$ ,  $P < 0.001$ ). P53 staining occurred in the majority of cases (78%) with most tumours showing a sporadic staining pattern with between 5 and 50% of cells staining in the specimen (Fig. 3.5.2.2). The degree of p53 intensity correlated significantly with the percentage of cells staining positively ( $r^2 = 0.667$ ,  $p < 0.001$ ).

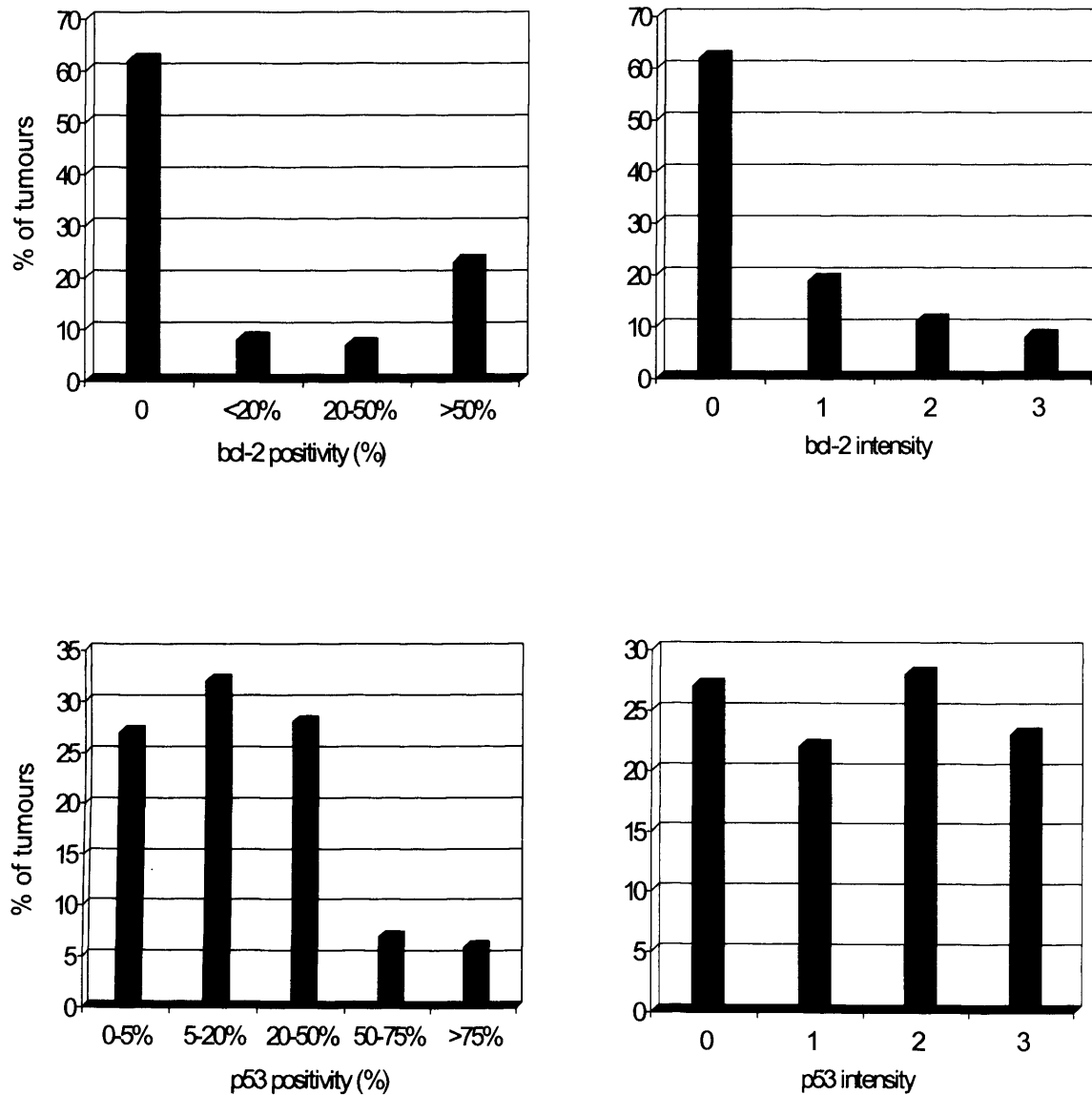
<b><u>Patient Characteristics</u></b>	<b>RT n=124</b>	<b>ACT I</b>	<b>CMT n=106</b>	<b>ACT I</b>
Age median	63	65	63	63
Sex M:F	47:53%	47:53%	38:62%	43:57%
Canal	103(83%)	218(77%)	86(81%)	213(73%)
Margin	21	67	20	79
T1	18(14%)	13%	15(14%)	13%
T2	48(39%)	37%	32(30%)	32%
T3	43(35%)	39%	45(43%)	40%
T4	15(12%)	11%	14(13%)	15%
Nodes impalpable	107(86%)	82%	86(81%)	77%
Nodes palpable N1	16		16	
N2	4		1	
<b><u>Response</u></b>				
Clinical response 6 weeks after initial treatment	CR >50% <50%	32(27%) 78(66%) 9(7%)	30% 62% 9%	36(36%) 59(59%) 5(5%)
Died before assessment	2	6	3	9
Missing	4		2	
<b><u>Deaths – 106</u></b>				
Anal	50(79%)		26(70%)	
Not anal	13		11	
<b><u>Grade</u></b>				
Well	28		26	
Moderate	72		58	
Poor	24		22	

**Table 3.1** A comparison of patient and tumour characteristics for RT alone and combined modality treatment between the ACT I trial and the present study



**Fig. 3.2** The distribution of proliferation markers





**Fig. 3.3** The distribution of apoptosis-related proteins

### 3.5.2.3 Cell cycle control markers: cyclin D1

Cyclin D1 is frequently overexpressed in other squamous cell carcinomas, yet only 18% of the anal carcinomas stained positively for this protein (Fig. 3.4). Interestingly the staining was both nuclear and cytoplasmic.

### 3.5.2.4 Hypoxia markers: Carbonic anhydrase IX

Carbonic anhydrase-9 is considered as a surrogate marker for hypoxia within tissues. Over 80% of anal carcinomas showed some degree of positivity for CA9 and high levels (>5%) were observed in approximately 50% of all tumours (Fig. 3.5).

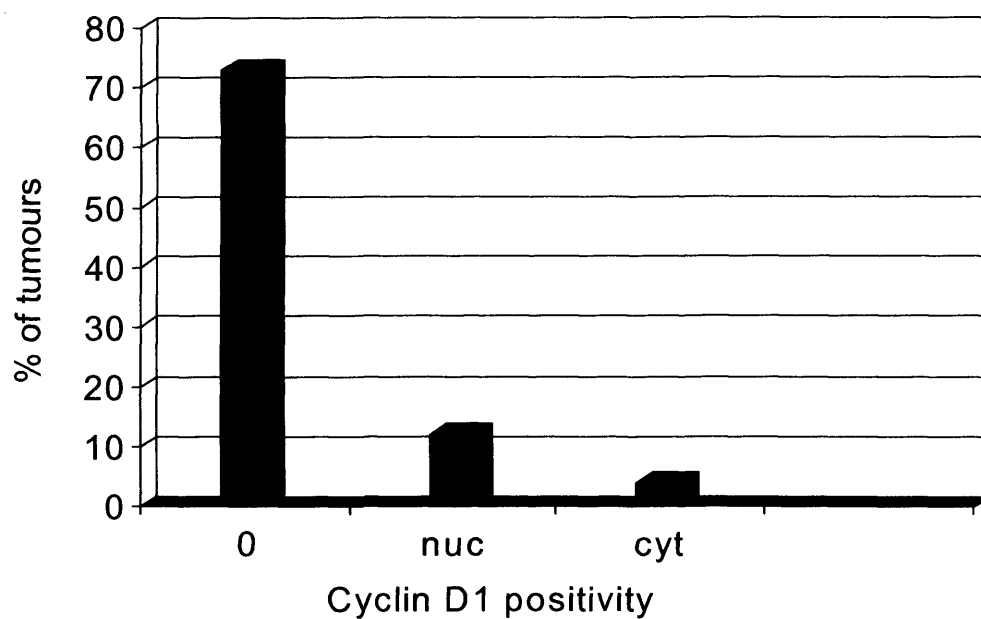
### 3.5.2.5 Vascular markers: CD34

CD34 expression was used to identify blood vessels within the tumours and the vascular density was calculated per mm<sup>2</sup>. Figure 3.6 shows the distribution of vascular densities among the tumours and the categorization of the data into quartiles for statistical analysis. The mean and median vascular densities were 78 and 74 vessels/mm<sup>2</sup> respectively; the range spanned from a low as 9 to 232 vessels/mm<sup>2</sup>.

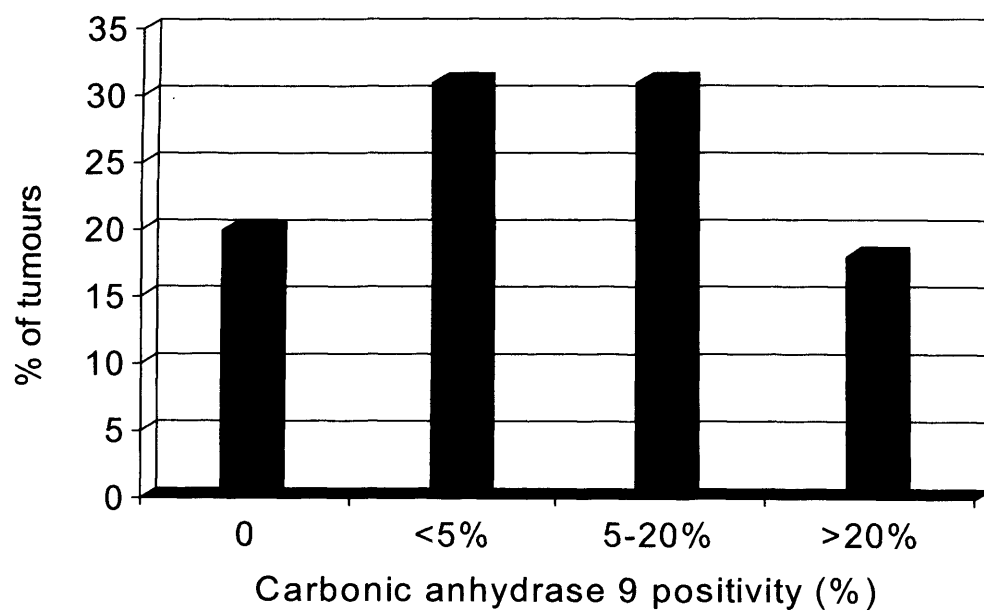
### 3.5.2.6 Markers of 5-FU response: TS, TP and DPD

Very few tumours were negative for TS and expression was widespread throughout each tumour. As a result only the intensity was scored. The majority of tumours showed weak to moderate intensity (Fig. 3.7.A). Over 93% of anal tumours stained positively for DPD. Almost half (47%) had greater than 50% of tumour cells staining positive (Fig. 3.7). The majority of staining however was weak. The DPD percentage score correlated with intensity of staining ( $r^2 = 0.589$ ,  $p < 0.001$ ).

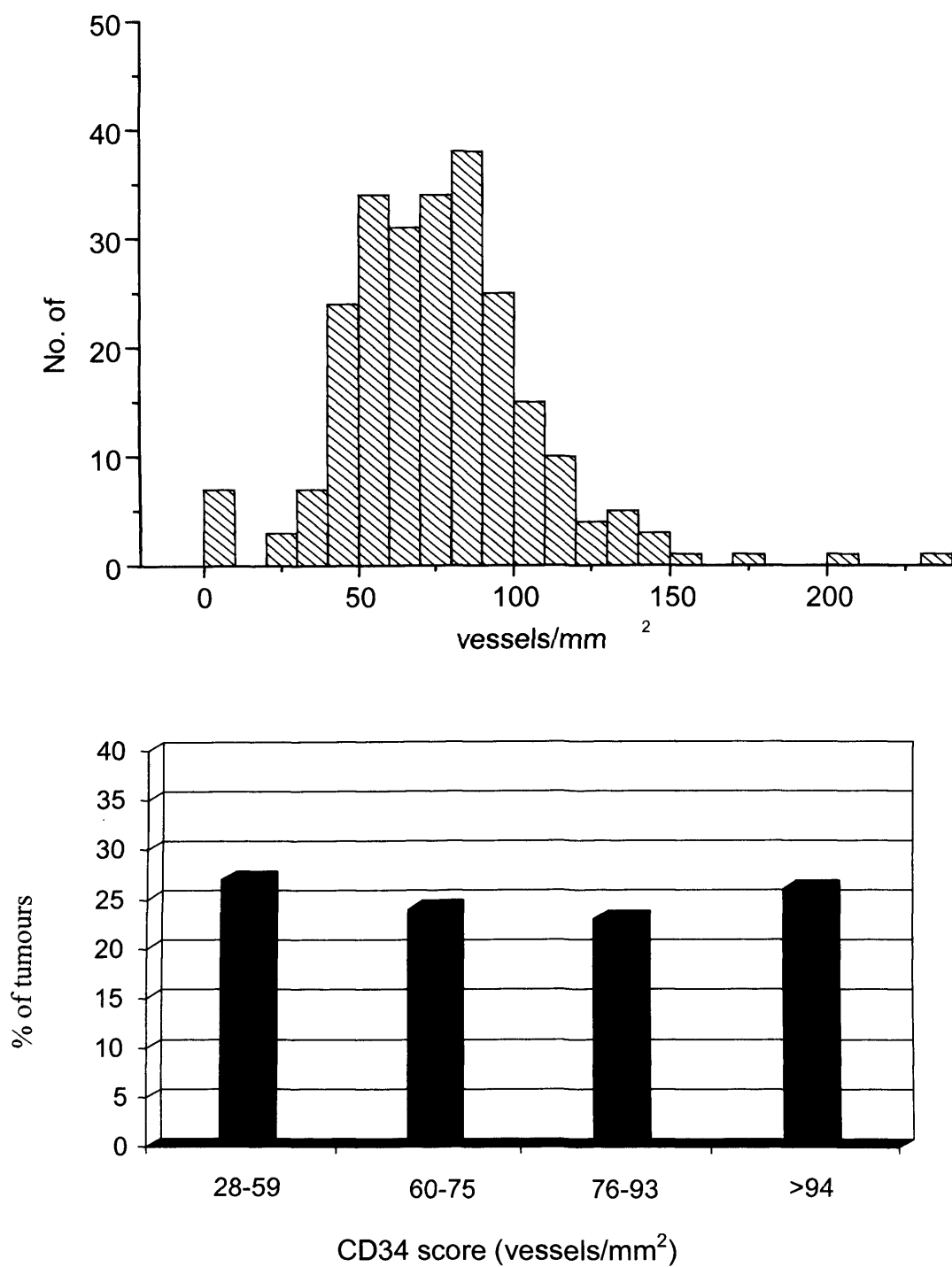
87% of cases stained positive for TP and staining occurred in the nucleus, stroma and cytoplasm (Fig. 3.8). Most samples stained positively in at least two areas. TP nuclear percentage correlated strongly with nuclear intensity ( $r^2 = 0.868$ ,  $p < 0.001$ ). TP cytoplasmic percentage also correlated strongly with cytoplasmic intensity ( $r^2 = 0.785$ ,  $p < 0.001$ ). There was also a positive correlation between the TP nuclear intensity score and the cytoplasmic percentage and intensity scores ( $r^2 = 0.539$ ,  $p < 0.001$ ,  $r^2 = 0.533$ ,  $p < 0.001$ ). Only 9% of cases stained for stroma alone. There were no correlations between the nuclear/cytoplasmic staining with stromal staining.



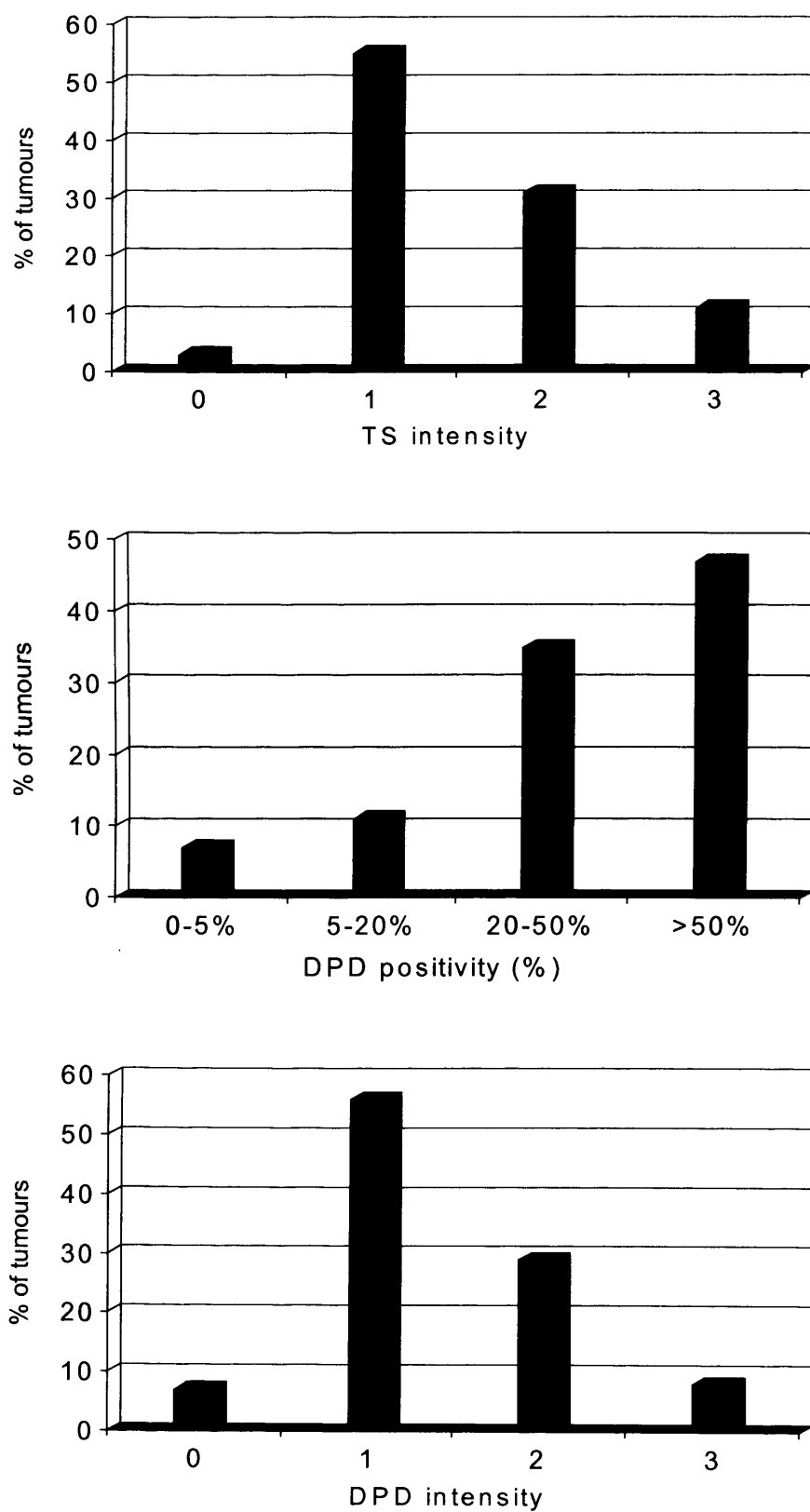
**Fig. 3.4** The distribution of cyclin D1 expression



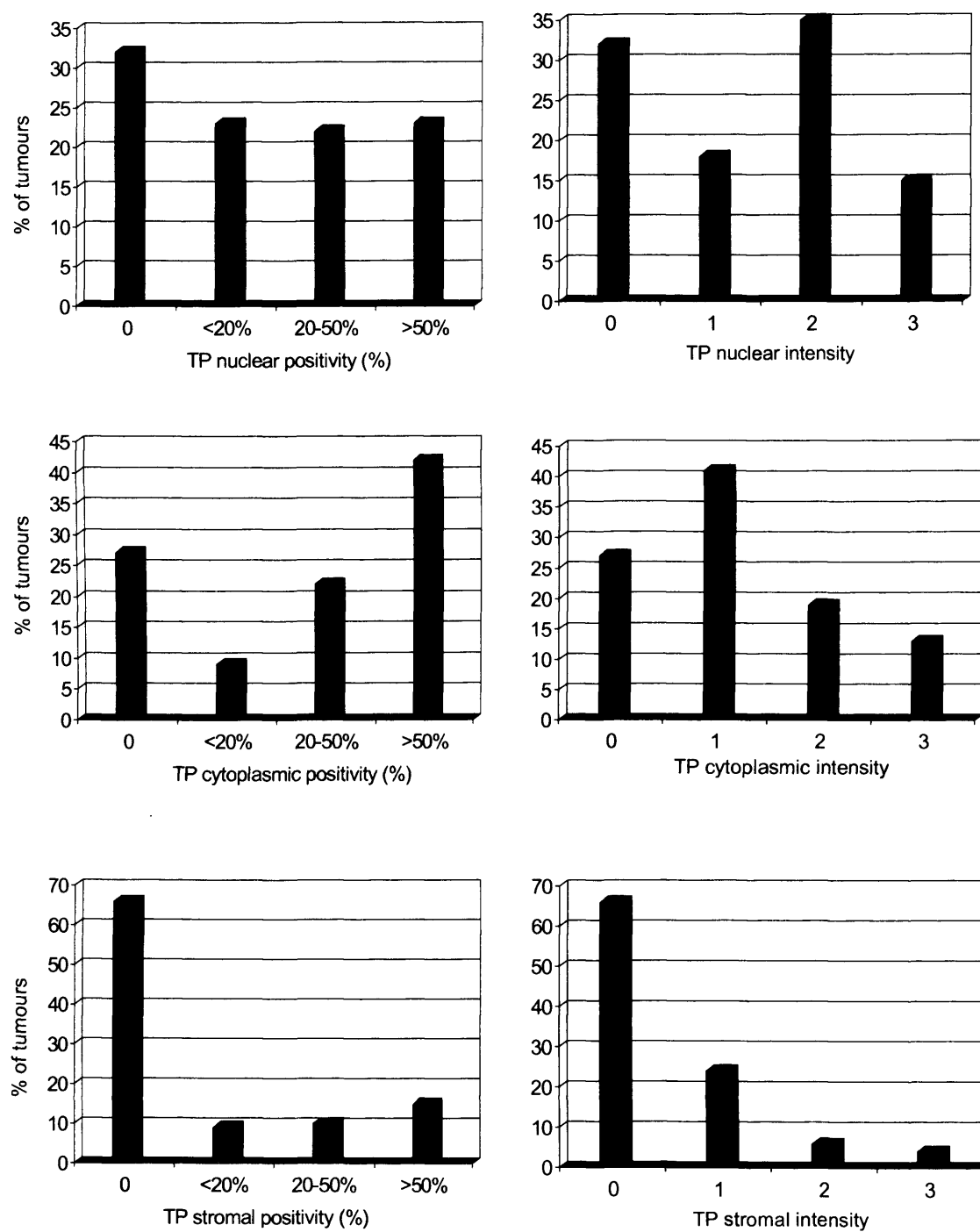
**Fig. 3.5** The distribution of carbonic anhydrase-9 expression



**Fig. 3.6** The distribution of vessel density between patients. The upper panel shows the overall distribution and the lower shows the data organized into quartiles



**Fig. 3.7** The distribution of TS and DPD



**Fig. 3.8** The distribution of TP expression. The molecular marker was present in the nucleus, cytoplasm and stroma of tumours and each localization was scored.

### 3.5.3 Correlation between markers and clinicopathological variables

As part of the trial database several clinicopathological variables were recorded. These included the sex of the patient, site of disease, and T and N stage. For the current series of patients, an experienced pathologist also classified the tumour grade.

Table 3.2, shows those significant correlations which were identified between the clinicopathological features and the biological markers. Only CA-9 expression, of the biological variables showed a correlation with increasing tumour stage. In contrast tumour grade correlated with several biological parameters including increased expression of bcl-2, Ki-67 and stromal expression of TP; negative correlations were noted with p53 and cytoplasmic TP expression. Anal tumours of male subjects had a higher expression of p53. Carcinomas of the anal canal correlated with an increased bcl-2, Ki-67 and cyclin A expression compared to anal margin tumours.

<u>Clinicopath</u>	<u>By variable</u>	<u>Spearman's Rank</u>	<u>P value</u>
T stage	N stage	0.218	0.001
T stage	Grade	0.159	0.015
T stage	CA9	0.150	0.022
Grade	Bcl-2% & Int	0.361/0.368	<0.001/<0.001
Grade	P53% & Int	-.218/-.194	0.048/0.003
Grade	Ki-67%	0.137	0.034
Grade	TS Int	0.191	0.003
Grade	TP Cyt Int	-.164	0.011
Grade	TP Str % & Int	0.217/0.228	0.001/<0.001
Sex: 1 male 2 female	P53% & Int	-.155/-.132	0.019/0.045
Site: 1 canal 2 margin	Bcl-2% & Int	-.163/-.170	0.013/0.010
Site	Ki-67%	-.166	0.012
Site	Cyc A%	-.158	0.017

**Table 3.2** Correlation between markers & clinical characteristics

### **3.5.4 Correlations between individual markers**

Several hypotheses were generated to look at the associations between some of the markers and determine their predominant role.

#### **3.5.4.1 Thymidylate synthase**

Thymidylate synthase is an enzyme involved in DNA synthesis. As a result it would be expected that TS would correlate with the proliferation markers. From the table below, see Table 3.3, it is clear that TS does correlate significantly with other markers of proliferation, Ki-67 and cyclin A.

There is also a positive correlation with bcl-2 intensity and percentage. This is consistent with the observation that TS expression is strongly correlated with grade and bcl-2 expression also correlates with grade. An increase in bcl-2 with increasing proliferation may be expected if cell death/loss mechanisms also show a proliferation-associated increase.

There was no correlation between TS and the other proteins thought to be associated with 5FU metabolism. There was also no association with p53, which has been hypothesized in other studies to modulate expression of TS [202]. The lack of correlation with markers of vascularity and hypoxia suggests that in this group of patients TS may purely be acting as a marker of DNA synthesis and not have a significant role in influencing the effect of 5FU.

#### **3.5.4.2 Dihydropyrimidine dehydrogenase**

DPD is the enzyme responsible for metabolizing 5FU. High tumour levels have been associated with an increased resistance to 5FU-based chemotherapy. In this analysis DPD did not appear to be associated with the other 5FU-dependent enzyme markers. However, there was an association with the markers of proliferation, hypoxia and vascularity, see Table 3.4.



<u>Variable</u>	<u>Spearman's</u>	<u>P-value</u>
Cyclin D	0.249	p=<0.001
Cyclin A%	0.290	p=<0.001
Cyclin A Int	0.313	p=<0.001
Ki67%	0.253	p=<0.001
Ki67 Int	0.197	p=0.002
Bcl2 Int	0.236	p=<0.001
Bcl2%	0.243	p=<0.001

**Table 3.3** Major correlations between TS expression and other molecular markers

<u>Variable</u>	<u>Spearman's</u>	<u>P value</u>
CA-9	0.171	0.008
CD34	-.139	0.032
Cyc A%	0.174	0.008
Cyc A Int	0.201	0.002
Ki67 Int	0.188	0.004
TP Cyt Int	0.158	0.015

**Table 3.4** Major correlations between DPD expression and other molecular markers

#### 3.5.4.3 Thymidine phosphorylase

TP is multi-faceted and is involved in DNA metabolism, increased tumour angiogenicity and increased intrinsic cancer cell aggressiveness. It activates capecitabine, an oral 5FU pro-drug and is also a marker of angiogenesis. TP is complicated by the fact that staining can occur within the tumour stroma, nucleus and cytoplasm. It is not clear whether in all of these sites TP's role is predominantly angiogenic. One hypothesis is that the stromal staining is most representative of the angiogenic activity and that nuclear staining may represent the form of TP involved in capecitabine activation.

From the earlier analysis, it was noted that cytoplasmic expression correlates with nuclear expression but neither of these correlate with the degree of stromal expression, suggesting a different role for cytoplasmic and nuclear TP from that of stromal TP. From Table 3.5, it can be seen that TP staining correlated with the expression of cyclin A irrespective of the cell or tissue localization probably reflecting its function in DNA metabolism. There was a positive correlation between nuclear and cytoplasmic TP expression and p53 but this was not observed with stromal TP expression. The association with p53 has been noted previously [203] suggesting that TP expression might be modulated by p53. Interestingly TP expression did not appear to correlate with vascularity nor the hypoxic marker CA-9. CD34 also did not correlate with CA-9.

#### 3.5.4.4 Proliferation markers

Cyclin A and ki67 were both strongly correlated with TS (see above) and each other ( $r^2=0.216$ ,  $p=0.001$ ), as expected. Cyclin A also correlated with p53 expression ( $r^2=0.142$ ,  $p=0.03$ ), which may purely be due to the cell-cycle dependence of each protein. There was also a positive correlation between cyclin A and increasing expression of CA-9 ( $r^2=0.134$ ,  $p=0.04$ ); this was not seen with Ki67.

<b><u>TP</u></b>	<b><u>By variable</u></b>	<b><u>Spearman's</u></b>	<b><u>P-value</u></b>
TP Nuc%	Cyc A% & Int	0.212/0.163	p=0.001/0.013
TP Nuc%	P53% & Int	0.193/0.169	p=0.003/0.009
TP Nuc Int	Cyc A% & Int	0.201/0.208	p=0.002/0.001
TP Nuc Int	P53% & Int	0.163/0.186	p=0.012/0.004
TP Cyt%	Cyc A% & Int	0.160/0.190	p=0.015/0.004
TP Cyt%	P53% & Int	0.139/0.174	p=0.033/0.007
TP Cyt Int	CycA Int	0.209	p=0.001
TP Cyt Int	P53% & Int	0.214/0.253	p=0.001/<0.001
TP Str%	Cyc A% & Int	0.158/0.188	p=0.016/0.004
TP Str Int	Cyc A Int	0.172	p=0.008

**Table 3.5** Major correlations between TP expression and other molecular markers

### 3.5.5 Survival analysis

The RFS rate at 5 years was estimated to be  $61\% \pm 5.2\%$  (1 standard error), (95% confidence intervals (CI) 50.8-71.2), in the chemoradiation arm and  $38.3\% \pm 4.6\%$ , (95% CI 29.3-47.3), in the radiotherapy alone arm. This difference in RFS between the two treatment groups was statistically significant on log rank testing  $p=0.0001$ . (Fig. 3.9).

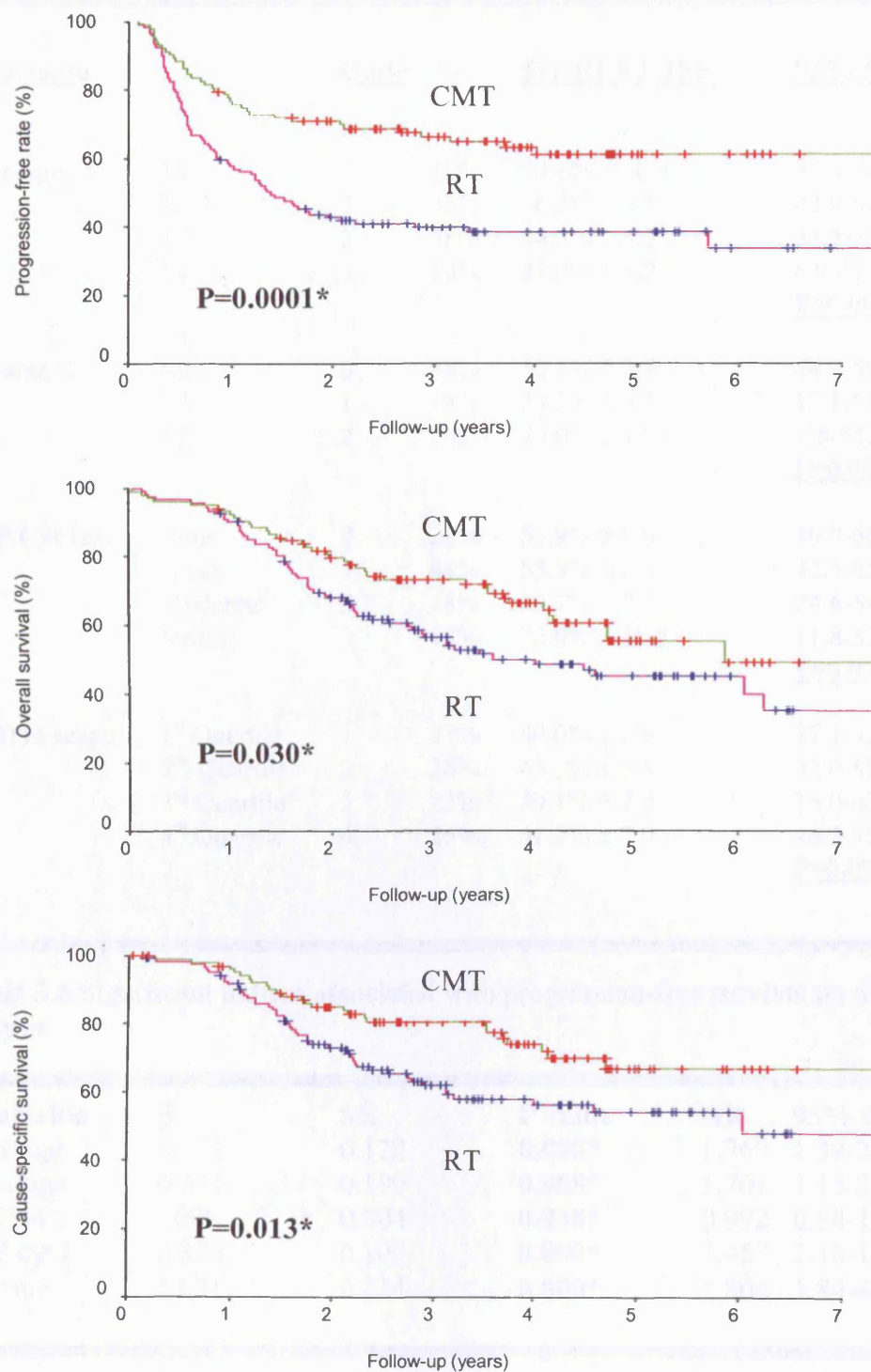
The overall survival rate between the two arms was  $55.4\% \pm 6.2\%$  (95% CI 43.2-67.6) for CMT and  $45.1\% \pm 5.2\%$  (95% CI 34.9-55.3), for RT at 5 years. This was statistically significant  $p=0.0301$ , see Figure 3.9, this was not significant in the original ACT I trial. When cause-specific survival (CSS) was analysed at 5 years the difference between the two treatment arms was more striking. The CSS for RT alone was  $54.1\% \pm 5.3\%$  (95% CI 43.7-64.5) and for the CMT arm  $66.9\% \pm 6.0\%$  (95% CI 55.1-78.7),  $p=0.0128$ . (Fig. 3.9).

#### 3.5.5.1 Progression –free survival

On univariate analysis, for the entire group, treatment arm, increasing T and N stage, and increasing TP cytoplasmic intensity were statistically associated with a poorer RFS. In the case of CD34 a decreasing score was significant for a poorer RFS rate at 5 years, see Table 3.6. Increasing p53 intensity was of borderline significance  $p=0.062$ .

Using the Cox proportional hazards model these factors remained significant on multivariate analysis, see Table 3.7.

Using this same model the markers and tumour indices were compared between the two treatment arms. In a univariate analysis increasing tumour T and N stage, TP cytoplasmic intensity, Ki-67 intensity and percentage, and CAIX score, were associated with an improved survival in the CMT arm. On multivariate analysis T stage, CD34 score and TP cytoplasmic intensity remained independently significant.



**Figure 3.9** Clinical outcome of the cohort of patients studied. The data are presented as progression-free (upper), overall (middle) and cause-specific survival (lower)

<u>Variable</u>		<u>Code</u>	<u>%</u>	<u>5yr RFS ± 1SE</u>	<u>95% CI</u>
<b>T stage:</b>	T1	1	14%	59.1% ± 10.8	37.9-80.3
	T2	2	35%	56.2% ± 6.3	43.9-68.5
	T3	3	38%	44.3% ± 5.5	33.5-55.1
	T4	4	13%	25.0% ± 8.2	8.9-41.
					<b><u>P=0.000*</u></b>
<b>N stage:</b>	N0	0	84%	52.1% ± 3.9	44.4-59.8
	N1	1	14%	34.2% ± 8.7	17.1-51.3
	N2	2	2%	20.0% ± 17.9	-15-55.
					<b><u>P=0.002*</u></b>
<b>TP Cyt Int:</b>	None	0	28%	53.9% ± 6.6	40.9-66.8
	Weak	1	41%	53.9% ± 5.8	42.5-65.3
	Moderate	2	18%	39.7% ± 7.7	24.6-54.8
	Strong	3	13%	32.0% ± 10.3	11.8-52.2
					<b><u>P=0.035*</u></b>
<b>CD34 score:</b>	1 <sup>st</sup> Quartile	1	27%	40.0% ± 6.6	27.1-52.9
	2 <sup>nd</sup> Quartile	2	24%	45.3% ± 6.8	32.0-58.6
	3 <sup>rd</sup> Quartile	3	23%	49.1% ± 7.2	35.0-63.2
	4 <sup>th</sup> Quartile	4	26%	61.9% ± 7.0	48.2-75.6
					<b><u>P=0.003*</u></b>

**Table 3.6** Significant indices associated with progression-free survival on univariate analysis

<b>Variable</b>	<b>β</b>	<b>SE</b>	<b>P value</b>	<b>HR</b>	<b>95% CI</b>
T stage	0.571	0.122	<b>0.000*</b>	1.769	1.39-2.25
N stage	0.531	0.199	<b>0.008*</b>	1.701	1.15-2.51
CD34	-.008	0.004	<b>0.038*</b>	0.992	0.98-1.00
TP cyt I	0.376	0.107	<b>0.000*</b>	1.457	1.18-1.80
Group	1.031	0.214	<b>0.000*</b>	2.804	1.84-4.26

**Table 3.7** Cox's proportional hazards model for significant indices on multivariate analysis with progression-free survival

SE = Standard error

HR = Hazard Ratio

CI = Confidence interval

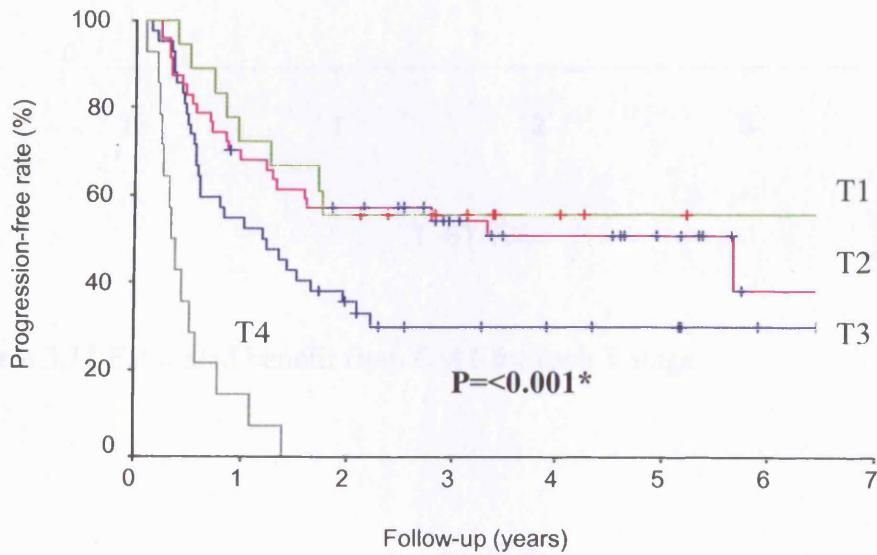
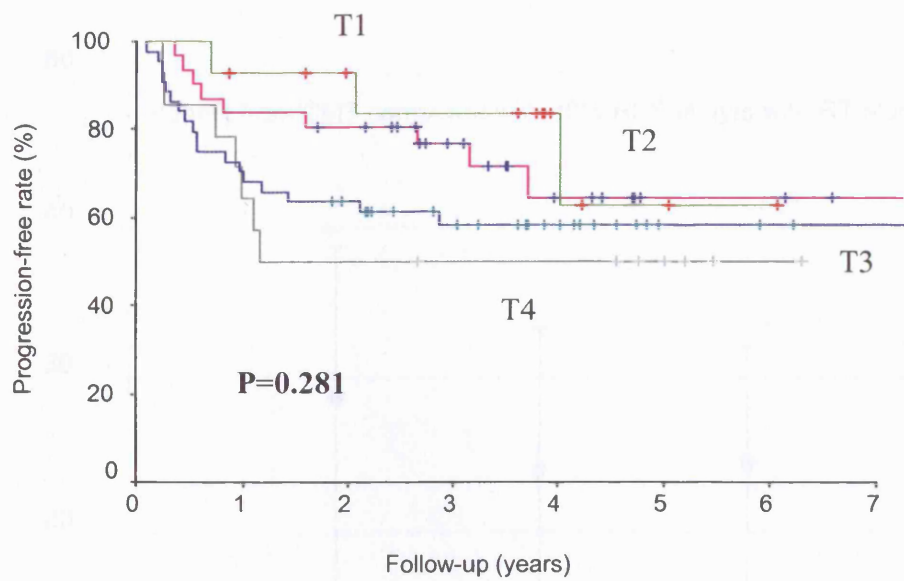
### 3.5.5.1.1 T stage

Increasing T stage was associated with an improved RFS in the CMT arm compared with a significantly poorer RFS in the RT alone treatment arm ( $\beta=0.383$ , SE 0.060,  $p<0.001$ , hazard ratio 1.466), see Figure 3.10. This might suggest that treatment with chemoradiation eliminates the prognostic effect of T stage. The finding that there was no significant difference in RFS between the different T stages for the CMT arm but it was significantly different for those patients having RT alone supports this hypothesis. In particular T4 tumours do very poorly with RT alone.

Table 3.8 shows the RFS for the two treatment arms according to T stage, and Figure 3.11 shows the estimated benefit from CMT over RT alone assuming a RFS rate of 40% at 5 years in the RT arm, as calculated in the survival analysis. This shows that patients with T4 tumours benefit most from CMT.

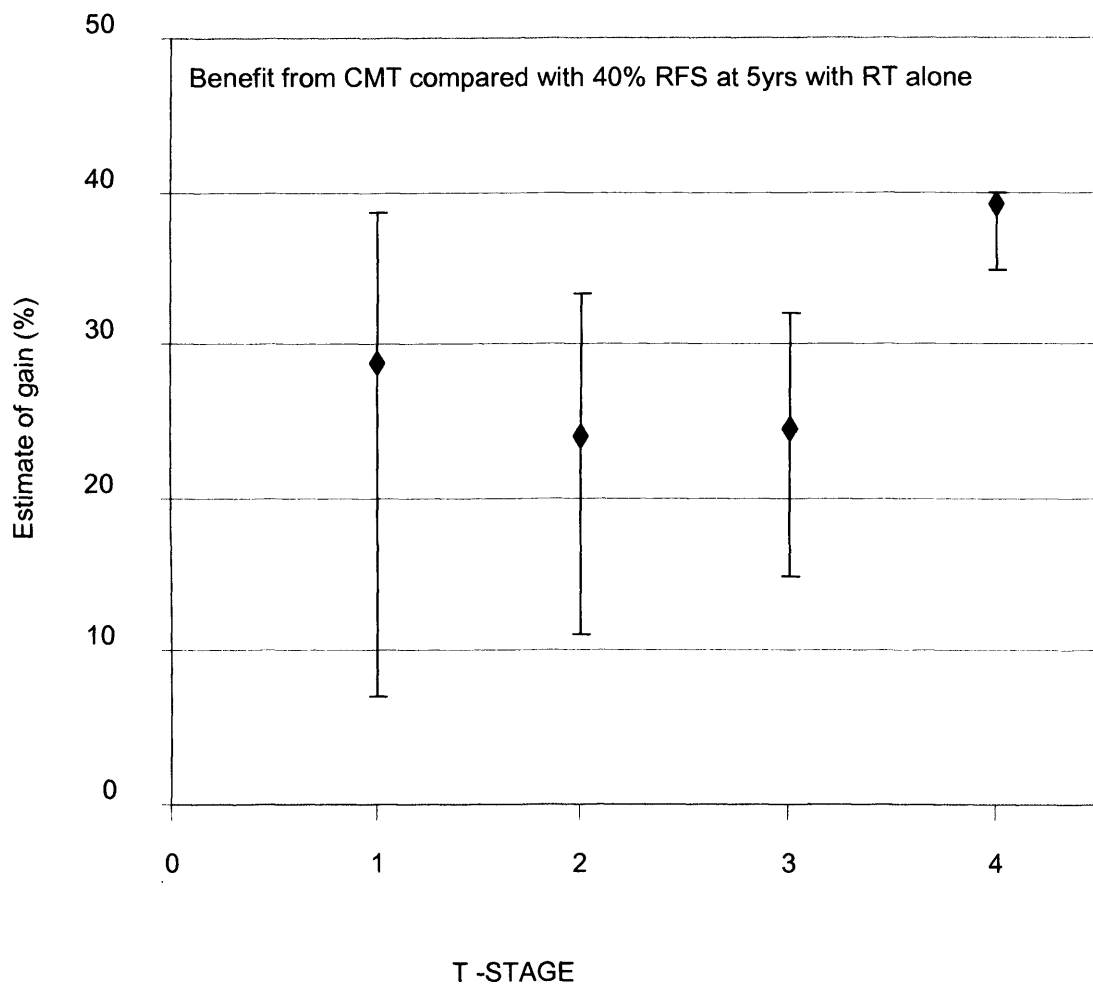
<u>T Stage</u>	<u>5 year relapse-free survival <math>\pm</math> 1SE</u>			
	<u>CMT</u>	<u>95% CI</u>	<u>RT</u>	<u>95% CI</u>
<b>T1</b>	62.7% $\pm$ 19.8	23.9-100	55.6% $\pm$ 11.7	32.7-78.5
<b>T2</b>	64.4% $\pm$ 10.4	44.0-84.8	50.6% $\pm$ 7.8	35.3-65.9
<b>T3</b>	58.1% $\pm$ 7.6	43.2-73.0	29.8% $\pm$ 7.2	15.7-43.9
<b>T4</b>	50.0% $\pm$ 13.4	23.7-76.3	All progressed	

**Table 3.8** 5 year RFS - T stage versus treatment arm



**Figure 3.10** Effect of T stage on progression-free survival; chemoradiation arm (upper) and RT alone arm (lower)





**Figure 3.11** Estimated benefit from CMT for each T stage

### 3.5.5.1.2 TP cytoplasmic intensity

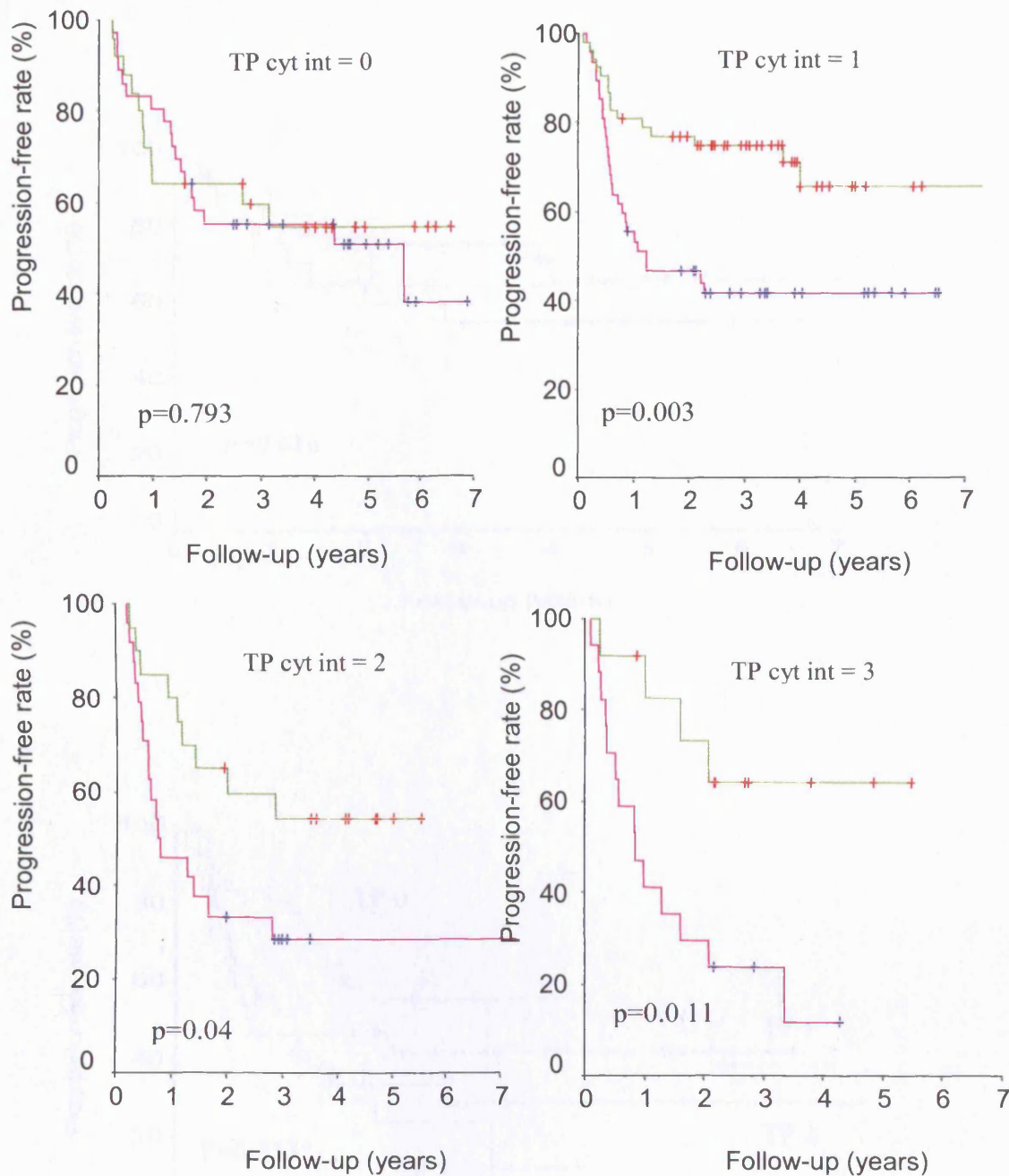
The cytoplasmic expression of TP was associated with an improved RFS in the CMT arm compared with a significantly poorer RFS in the RT alone treatment arm ( $\beta=0.226$ , SE 0.056,  $p<0.001$ , hazard ratio 1.254), see Figure 3.12. The influence was absent in those tumours with low expression of TS in the cytoplasm.

These results suggest that with chemoradiation treatment the effect of TP cytoplasmic intensity is not as important with regards to prognosis as it is with RT alone, in a similar way to the effect of T stage. This is demonstrated in Figure 3.13, where there is no significant difference in progression-free rate between the different TP expressing tumours for the CMT arm, however it is significantly different for those patients having RT alone.

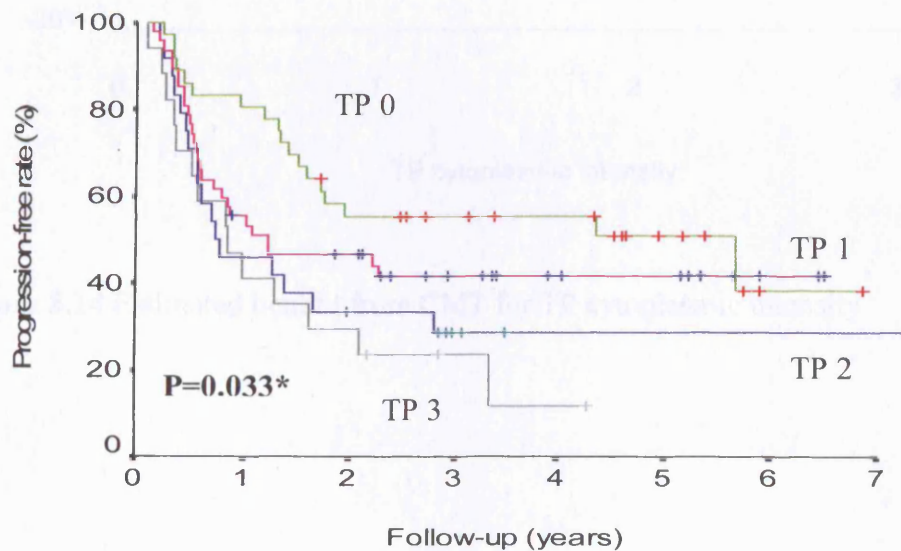
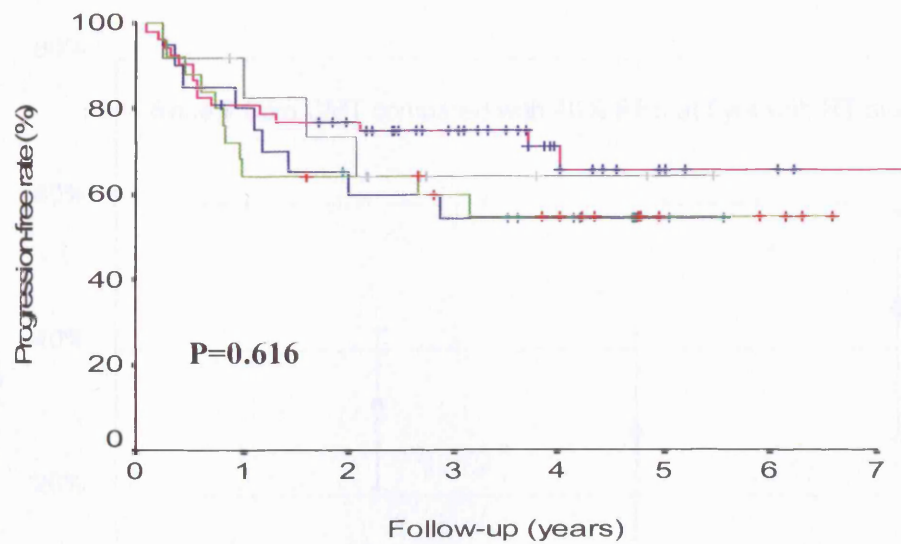
Table 3.9 shows the RFS for the two treatment arms according to increasing TP cytoplasmic intensity, and Figure 3.14 shows the estimated benefit from CMT over RT alone, once again assuming a RFS rate of 40% at 5 years in the RT arm, as calculated in the survival analysis.

<u>T P cyt int</u>	<u>5 year relapse-free survival <math>\pm</math> 1SE</u>			
	<u>CMT</u>	<u>95% CI</u>	<u>RT</u>	<u>95%CI</u>
TP = 0	54.5% $\pm$ 10.3	34.3-74.7	50.6% $\pm$ 8.8	33.4-67.8
TP = 1	65.3% $\pm$ 8.3	49.0-81.6	41.3% $\pm$ 7.4	26.8-55.8
TP = 2	54.2% $\pm$ 11.3	32.1-76.3	28.6% $\pm$ 9.4	10.2-47.0
TP = 3	64.2% $\pm$ 14.4	36.0-92.4	11.8% $\pm$ 9.8	-7.4-31.0

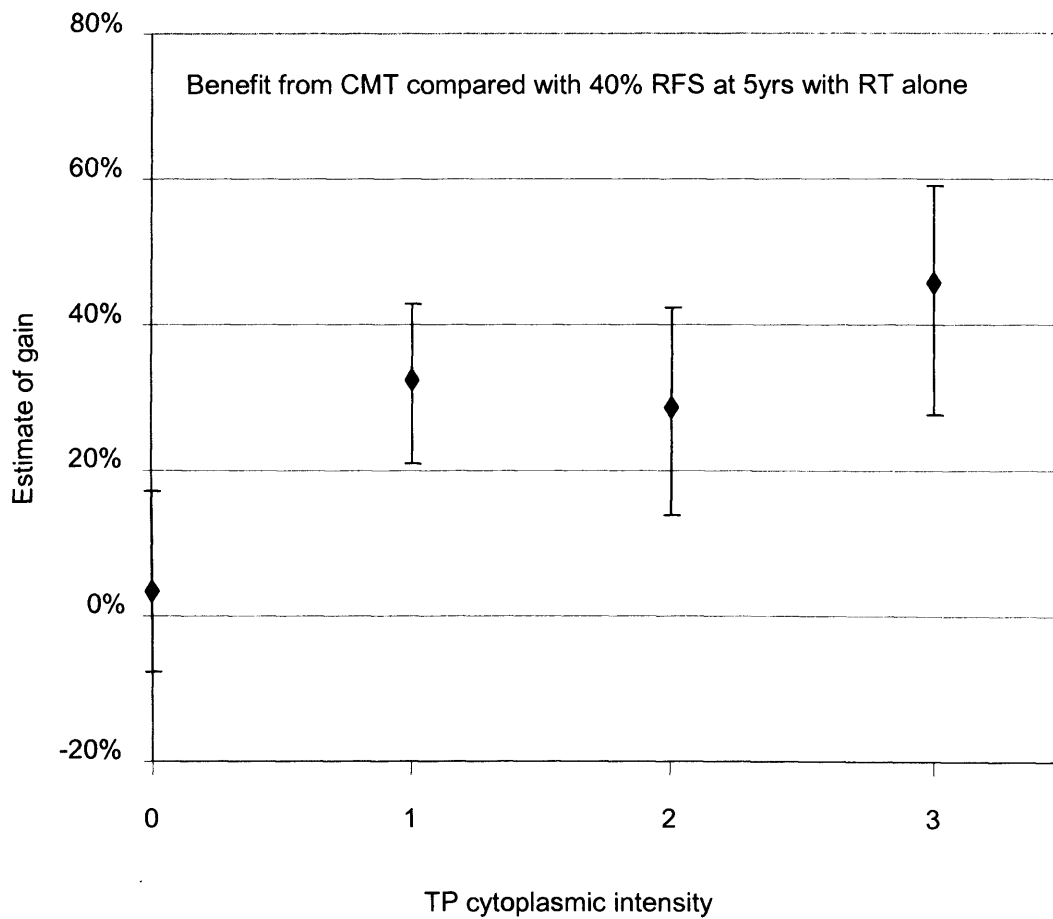
**Table 3.9** 5 year RFS - TP cytoplasmic intensity versus treatment arm



**Fig. 3.12** The influence of cytoplasmic expression of TP on the outcome of ACT I trial patients. The green lines are those treated with combined chemoradiation and the pink are those treated by radiation alone.



**Figure 3.13** The influence of treatment arm on the clinical significance of cytoplasmic TP expression. The upper panel shows those patients treated by chemoradiation, whilst the lower represents radiation alone. The lines in both panels follow the same colours.



**Figure 3.14** Estimated benefit from CMT for TP cytoplasmic intensity

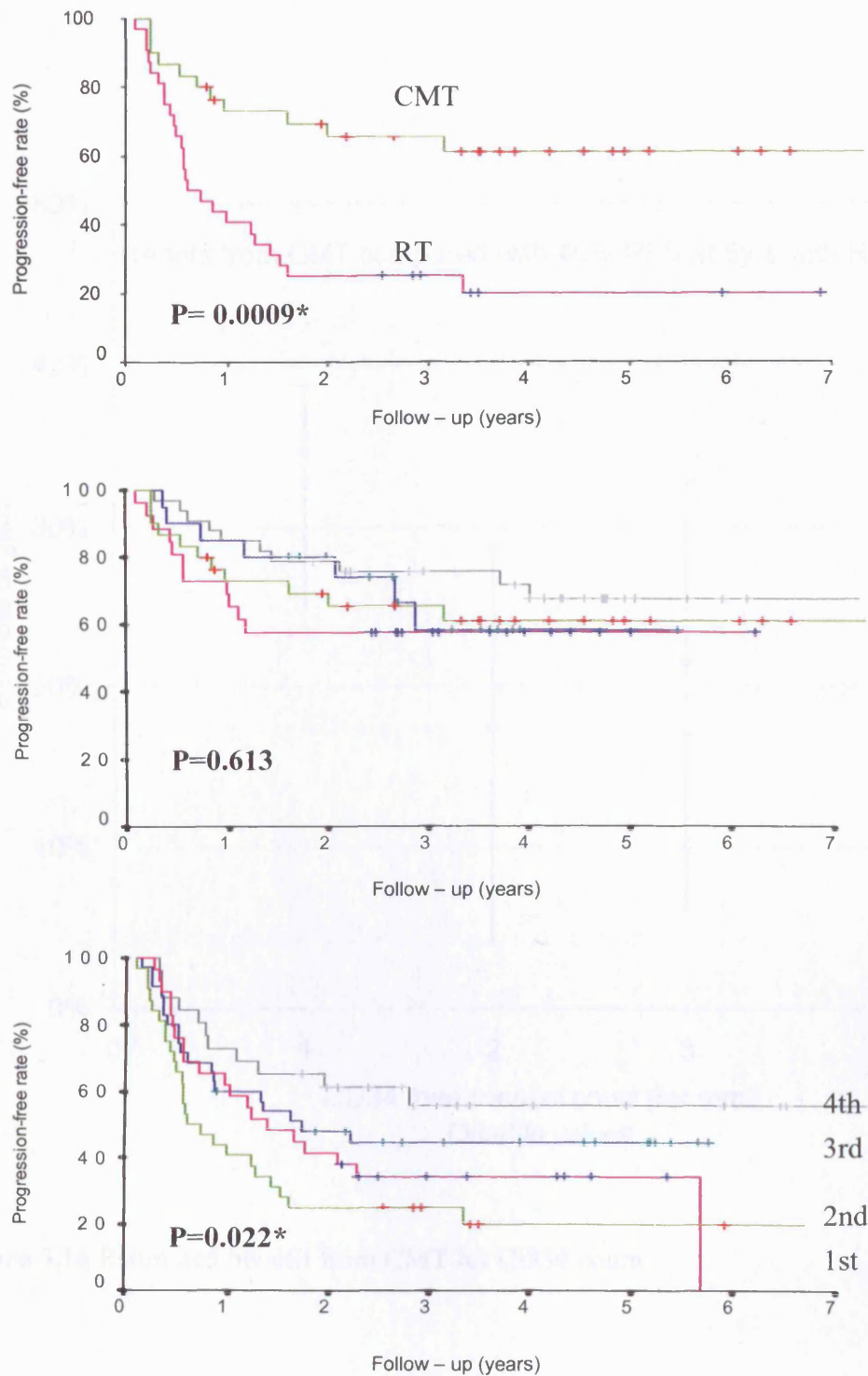
### 3.5.5.1.3 CD34

A decreasing CD34 ( $\beta = -.005$ , SE 0.002,  $p = 0.014$ , hazard ratio 0.995) was associated with an improved RFS in the CMT treatment arm compared to RT alone. This was only significant however, for those tumours where the CD34 count was in the first quartile, see Figure 3.15. Tumours that express a low CD34 score in the first quartile benefit significantly from combined modality treatment. In a previous study in rectal tumours [108] a low micro-vessel density was prognostic for increased tumour recurrence.

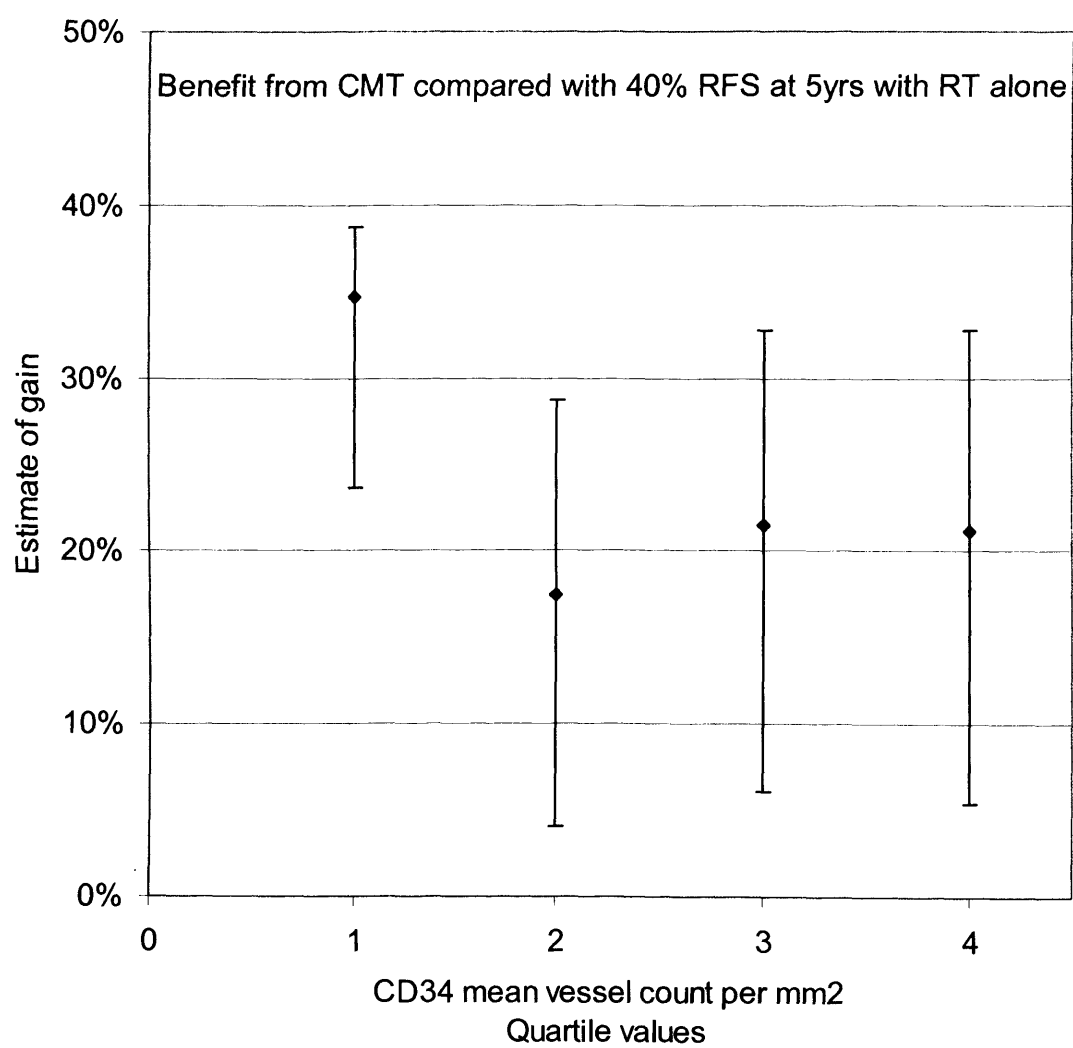
These results suggest that with chemoradiation treatment the effect of a decreasing CD34 score is not as important with regards to prognosis as it is with RT alone. This is demonstrated in Figure 3.15, where there is no significant difference in progression-free rate between the different CD34 quartiles for the CMT arm, however it is significantly different for those patients receiving RT alone. RFS in the RT arm significantly improved with increasing CD34 count, see Table 3.10. Figure 3.16 shows the estimated benefit from CMT over RT alone.

<u>CD34</u>	<u>5 year relapse-free survival <math>\pm</math> 1SE</u>			
	<u>CMT</u>	<u>95% CI</u>	<u>RT</u>	<u>95% CI</u>
1 <sup>st</sup> Quartile	61.0% $\pm$ 9.3	42.8-79.2	20.0% $\pm$ 7.6	5.1-34.9
2 <sup>nd</sup> Quartile	57.7% $\pm$ 9.7	38.7-76.7	34.1% $\pm$ 8.9	16.7-51.5
3 <sup>rd</sup> Quartile	58.2% $\pm$ 12.7	33.3-83.1	44.6% $\pm$ 8.6	27.7-61.5
4 <sup>th</sup> Quartile	67.4% $\pm$ 8.6	50.5-84.3	55.7% $\pm$ 10.2	35.7-75.7

**Table 3.10** 5 year RFS stratified by CD34 count and treatment arm



**Figure 3.15** The influence on vascularity on the outcome of patients treated in the ACT I trial. The upper panel compares tumours within the lowest vascular score (28-59 vessels/mm<sup>2</sup>) between the two arms of the trial. The middle panel shows the influence of differing vascularity in the chemoradiation arm and the lower panel demonstrates the clinical significance of vascularity in the radiation alone arm. The middle and lower panel use the same colours and lines.



**Figure 3.16** Estimated benefit from CMT for CD34 count



### **3.5.5.2 Cause-specific survival**

On univariate analysis, RT treatment arm, increasing tumour T and N stage, increasing P53 expression and a decreasing CD34 score were significant for a poorer CSS, see Table 3.11.

Using the Cox proportional hazards model these indices remained independently significant on multivariate analyses, except for CD34, see Table 3.12.

As would be expected an increasing T and N stage and RT treatment arm indicates a poorer CSS as seen in the RFS data. However, an increasing P53 intensity is also significant for a poorer CSS, which was not seen for RFS.

#### **3.5.5.2.1 P53**

A decreasing P53 ( $\beta = -.273$ , SE 0.138,  $p = 0.048$ , hazard ratio 0.761) was associated with a significantly improved CSS in the CMT treatment arm compared to RT alone. This was only significant however, for those tumours, which were negative for P53, see Figure 3.17.

CSS in the both treatment arms worsened with increasing P53 intensity. This demonstrates that P53 remains an independent prognostic factor irrespective of the treatment received.

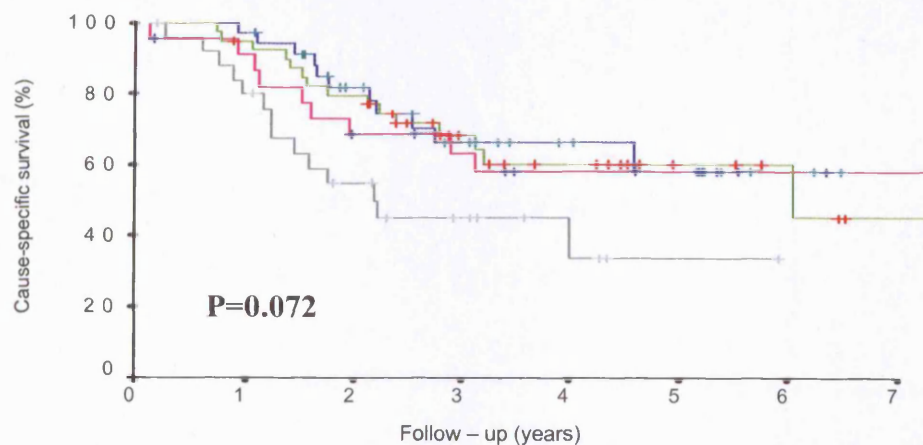
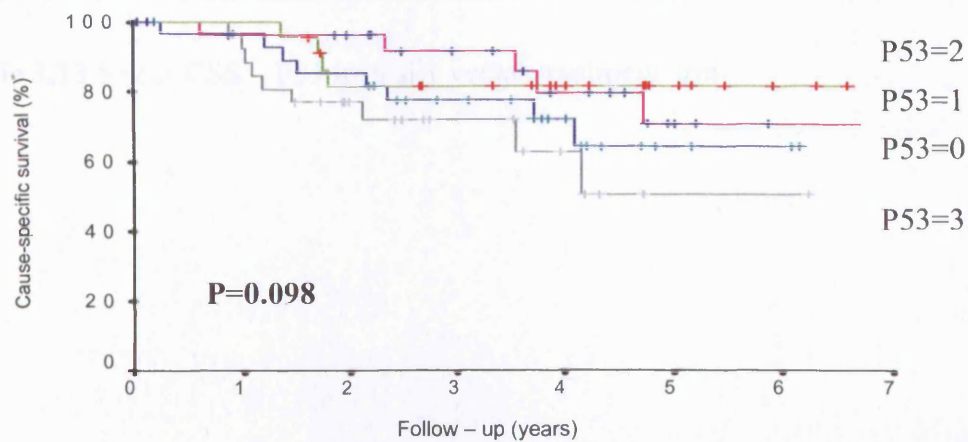
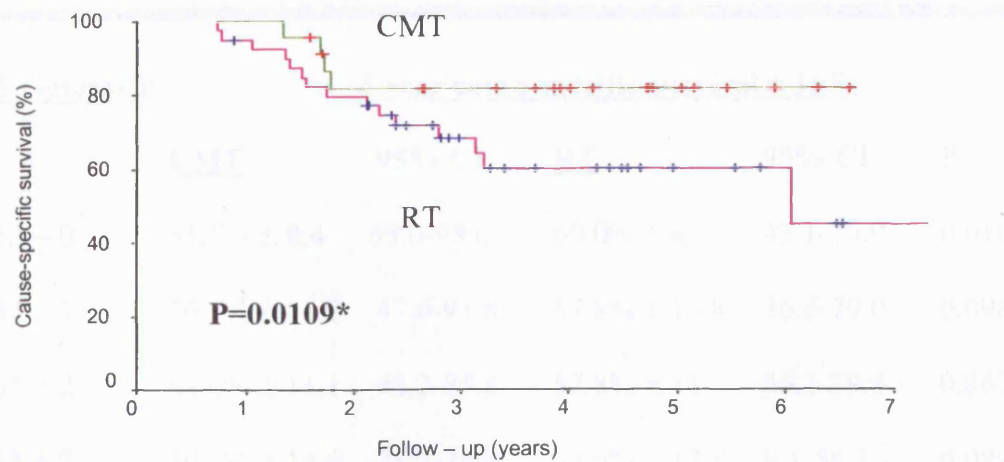
Table 3.13 shows the CSS for the two treatment arms according to increasing P53 intensity, and Figure 3.18 shows the estimated benefit from CMT over RT alone.

<b>Variable</b>	<b>Code</b>	<b>%</b>	<b>5yr RFS <math>\pm</math> 1SE</b>	<b>95% CI</b>
<b>Site:</b>	Canal	1	84%	55.9% $\pm$ 4.5
	Margin	2	16%	81.2% $\pm$ 6.5
				<b><u>P=0.053*</u></b>
<b>T stage:</b>	T1	1	14%	59.7% $\pm$ 12.9
	T2	2	35%	73.0% $\pm$ 6.5
	T3	3	38%	56.5% $\pm$ 6.2
	T4	4	13%	31.2% $\pm$ 9.0
				<b><u>P=0.000*</u></b>
<b>N stage:</b>	N0	0	84%	64.1% $\pm$ 4.3
	N1	1	14%	37.2% $\pm$ 10.6
	N2	2	2%	40.0% $\pm$ 21.9
				<b><u>P=0.003*</u></b>
<b>P53 Int:</b>	None	0	27%	68.6% $\pm$ 6.3
	Weak	1	22%	65.1% $\pm$ 7.9
	Moderate	2	28%	59.6% $\pm$ 8.3
	Strong	3	23%	42.0% $\pm$ 9.7
				<b><u>P=0.030*</u></b>
<b>CD34 score:</b>	1 <sup>st</sup> Quartile	1	27%	49.4% $\pm$ 7.6
	2 <sup>nd</sup> Quartile	2	24%	57.7% $\pm$ 7.8
	3 <sup>rd</sup> Quartile	3	23%	62.1% $\pm$ 8.1
	4 <sup>th</sup> Quartile	4	26%	71.5% $\pm$ 7.8
				<b><u>P=0.009*</u></b>

**Table 3.11** Significant indices associated with cause-specific survival on univariate analysis

<b>Variable</b>	<b><math>\beta</math></b>	<b>SE</b>	<b>P value</b>	<b>HR</b>	<b>95% CI</b>
T stage	0.713	0.150	<b>0.000*</b>	2.040	1.52-2.73
N stage	0.553	0.210	<b>0.008*</b>	1.739	1.15-2.62
P53 Int	0.244	0.107	<b>0.021*</b>	1.277	1.03-1.57
Group	0.849	0.218	<b>0.000*</b>	2.337	1.52-3.58

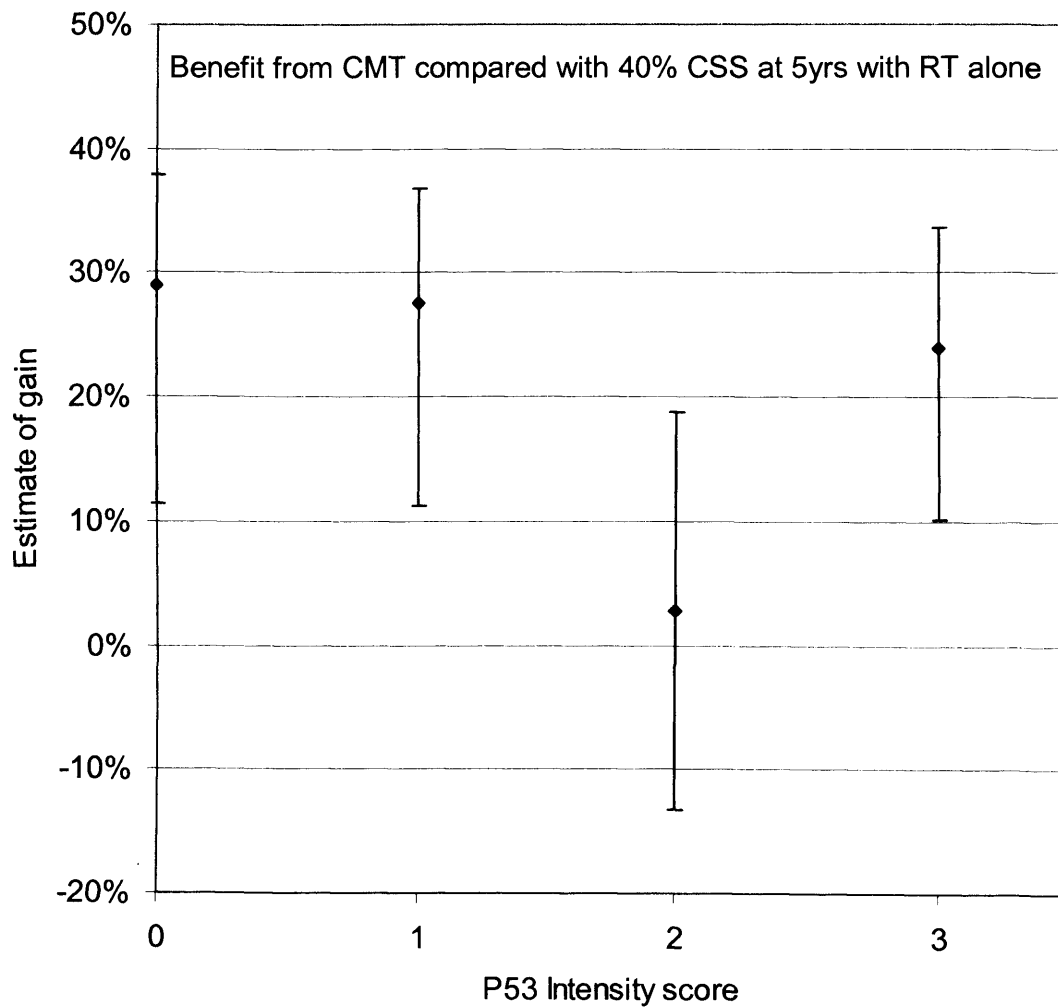
**Table 3.12** Cox's proportional hazards model for significant indices on multivariate analysis with progression-free survival



**Figure 3.17** The influence of p53 on CSS. The upper panel compares tumours within the lowest p53 score between the two arms of the trial. The middle panel shows the influence of differing p53 in the chemoradiation arm and the lower panel demonstrates the clinical significance of p53 in the radiation alone arm. The middle and lower panel use the same colours and lines.

<b><u>P53 intensity</u></b>	<b><u>5 year cause-specific survival <math>\pm</math> 1SE</u></b>				
	<b><u>CMT</u></b>	<b>95% CI</b>	<b><u>RT</u></b>	<b>95% CI</b>	<b>P</b>
P53 = 0	81.5% $\pm$ 8.4	65.0-98.0	60.0% $\pm$ 8.6	43.1-76.9	0.010
P53 = 1	70.7% $\pm$ 11.8	47.6-93.8	57.8% $\pm$ 10.8	36.6-79.0	0.098
P53 = 2	64.0% $\pm$ 11.1	42.2-85.8	57.8% $\pm$ 11	36.2-79.4	0.867
P53 = 3	50.3% $\pm$ 14.6	21.7-78.9	33.6% $\pm$ 12.5	9.1-58.1	0.089

**Table 3.13** 5 year CSS – P53 intensity versus treatment arm



**Figure 3.18** Estimated benefit from CMT for P53 intensity

### **3.5.5.3 Response to chemoradiation**

For the purpose of this analysis response was divided into three subgroups. These were complete response, >50% response and <50% response or progressive disease. Only one patient progressed during treatment, so they were grouped with the <50% responders.

#### **3.5.5.3.1 Survival analysis**

RFS and CSS were analysed for the different response groups. This showed a significant difference at 5 years for both parameters for each group. See Table 3.14. Figure 3.19 clearly demonstrates the importance of a good response to treatment in achieving a prolonged survival.

#### **3.5.5.3.2 Correlation with other parameters**

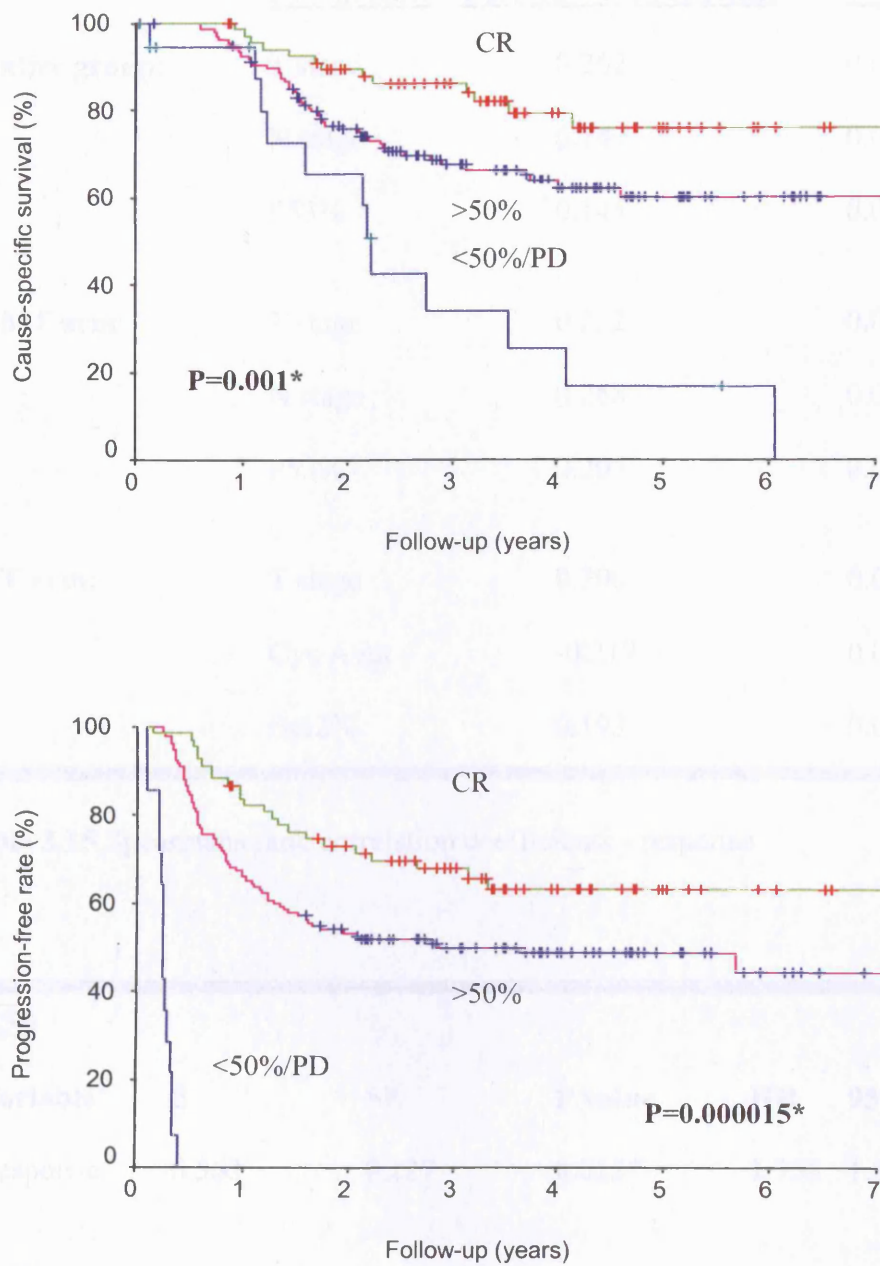
For the entire study group, using Spearman's Correlation Coefficient, significant associations were found between a poorer response and increasing T and N stage and p53 percentage of cells stained, see Table 3.15. In the CMT alone group the same significant associations were found. However, in the RT treatment group, increasing T stage, decreasing cyclin A intensity, increasing bcl2% and intensity correlated with a poorer response to radiation.

#### **3.5.5.3.3 Response as a prognostic marker**

If response is analysed as an indices in the Cox proportional hazards model it is an independent, highly significant prognostic indicator marker for both RFS and CSS. See Table 3.16.

<b><u>Response</u></b>	<b><u>5 year CSS <math>\pm</math> 1SE</u></b>	<b><u>95% CI</u></b>
Complete	75.6% $\pm$ 6.3	63.3-87.9
>50%	60.1% $\pm$ 5.0	50.3-69.9
<50%/ PD	17.0% $\pm$ 10.8	-4.2-38.2
	<b><u>5 year RFS <math>\pm</math> 1SE</u></b>	<b><u>95% CI</u></b>
Complete	63.0% $\pm$ 6.3	50.7-75.3
>50%	48.5% $\pm$ 4.4	39.9-57.1
<50%/ PD	None at 5 years	

**Table 3.14** RFS & CSS association with response to treatment



**Figure 3.19** Effect of clinical response to treatment on cause-specific survival (upper); and progression-free survival (lower)



	<u>By Variable</u>	<u>Correlation Coefficient</u>	<u>P value</u>
<b>Entire group:</b>	T stage	0.262	0.000*
	N stage	0.149	0.026*
	P53%	0.145	0.030*
<b>CMT arm:</b>	T stage	0.232	0.018*
	N stage	0.268	0.006*
	P53%	0.207	0.036*
<b>RT arm:</b>	T stage	0.306	0.001*
	Cyc A int	-0.217	0.018*
	Bcl2%	0.193	0.034*

**Table 3.15** Spearman's rank correlation coefficients - response

<u>CSS</u>					
<b>Variable</b>	<b><math>\beta</math></b>	<b>SE</b>	<b>P value</b>	<b>HR</b>	<b>95% CI</b>
Response	0.563	0.227	<b>0.013*</b>	1.755	1.13-2.74
<u>RFS</u>					
Response	1.221	0.278	<b>0.000*</b>	3.392	1.97-5.85

**Table 3.16** Response as an independent prognostic indicator of both survival outcomes on multivariate analysis

## 3.6 Discussion

Carcinoma of the anus is primarily a loco-regional disease with a low rate of distant metastasis [13, 204]. Adequate control of the primary site is therefore extremely important, to optimise the chances of cure while, if possible, preserving anorectal function. With current chemoradiation and surgical techniques locoregional failure still occurs in up to 35% of patients [15, 16]. Unfortunately, most patients with tumours that fail to respond to chemoradiation will die of their disease [205, 206].

To improve upon the current situation it is becoming increasingly important to identify other factors which may help in predicting response to treatment and clinical outcome; the use of biological markers is an area receiving growing attention in many cancers. It is important to determine which pre-treatment factors are predictive of patients' responses to chemoradiation. This could help identify patients who need more aggressive treatment with a more intensive chemoradiation regimen or possibly a primary surgical approach.

There have been few studies investigating the role of molecular markers in the management of patients with anal carcinoma. To date, indicators of patient outcome have been derived from clinicopathological features including tumour stage/size, extent of lymph node involvement and anatomical site [15, 207, 208]. However, despite careful assessment of these features it is still difficult to accurately predict outcome in individual patients with this disease.

In this current series of 230 anal carcinomas, a panel of 10 molecular markers were studied. The aim was to identify potential markers which could predict outcome and response to treatment independently of the clinicopathological factors. Markers were stained for intensity and percentage of cells staining positive. In all cases the intensity scoring correlated significantly with percentage, in each of the molecular markers.

### 3.6.1 Correlation between molecular markers and clinicopathological variables

The first part of the study was to analyse the association between the molecular markers and the clinicopathological characteristics. This demonstrated that an

increasing T stage was associated with an increasing N stage, as might be expected. Increasing T stage also correlated with grade and CA9, implying that the larger, faster growing tumours were of a higher grade and more hypoxic. This has also been demonstrated in a study of [191] non-small cell lung carcinomas. In this series, grade also correlated with increasing Ki-67 and bcl-2, indicating the degree of proliferation correlated with a higher grade. This may explain the positive correlation of Ki-67 and bcl-2 with carcinomas of the anal canal, because these are usually of a higher grade than margin carcinomas. This association between grade and Ki67 has also been demonstrated in breast carcinomas [209].

Grade also positively correlated with TS, a marker of 5FU metabolism and DNA synthesis, and with the stromal expression of the angiogenic factor TP. However, there was a negative correlation with tumour TP expression. This variation in association of grade with different TP locations has been noted previously [210] in both breast carcinomas and bladder carcinomas [211]. Grade negatively correlated with p53 staining, indicating that the well differentiated tumours expressed higher levels of p53. This association is illustrated in the correlation between sex and p53. Lower levels of p53 positivity were seen in females, who are known to have a higher incidence of anal canal tumours, which are usually more poorly differentiated than those found at the anal margin.

In this series there was no association between DPD and any of the clinicopathological factors, in particular grade and stage. This is interesting because more data is becoming available on the relevance of DPD expression in tumours detected by IHC. In a study [212] of bladder carcinomas, the DPD level paralleled the tumour stage and grade and activity of DPD was 2 fold higher in carcinoma tissues compared to normal tissues. The activity was also 2 fold higher in invasive carcinomas compared to superficial bladder cancers. This suggests that the level of DPD might be associated with the malignant potential. This higher level of DPD in tumour tissues has also been found in head and neck cancers with a 6.6 fold increase [75] and in colorectal carcinomas with a 3.3 fold increase [213]. This high DPD activity in the cancer tissues compared with normal tissues may contribute to the unfavourable differential between the anticancer effect and the adverse effect of 5FU.

A higher degree of degradation may occur in cancer tissues compared to normal tissues.

### 3.6.2 Correlations between molecular markers

The role and expression of molecular markers in anal carcinoma remains largely unstudied. In this series there was a high proliferation index and expression of p53, CA9, and markers of 5FU metabolism DPD, TP and TS. Over a third expressed bcl-2, however only 18% stained positively for cyclin D1, in other series this figure has been much higher at 50-80% in breast and head and neck cancers [149, 214].

The significant associations between the molecular markers were studied. Ki-67 and cyclin A strongly correlated as markers of proliferation and as might be expected they also correlated with TS, a marker of DNA synthesis. TS also correlated positively with bcl-2 intensity and percentage. This is consistent with the observation that both TS and bcl-2 expression correlated strongly with grade. An increase in bcl-2 with increasing proliferation may be expected if cell death/loss mechanisms also show a proliferation-associated increase. There was no correlation between TS and the other proteins thought to be associated with 5FU metabolism. There was also no association with p53, which has been hypothesized in other studies to modulate expression of TS [202]. The lack of correlation with markers of vascularity and hypoxia suggests that in this group of patients TS may purely be acting as a marker of DNA synthesis and not have a significant role in influencing the effect of 5FU. TS did however, correlate strongly with cyclin D1. This may well be due to the expression of both these proteins predominantly at G1 into S phase. Equally overexpression of cyclin D1 shortens the G1 phase and reduces dependence on growth factors [215], which in turn may result in loss of cell cycle control and increased cell proliferation. As a result TS may increase as a marker of proliferation.

TP cytoplasmic expression correlated with nuclear expression but neither of these correlated with the degree of stromal expression, suggesting different roles. TP staining correlated with the expression of cyclin A irrespective of the cell or tissue localization probably reflecting its function in DNA metabolism. There was a positive correlation between nuclear and cytoplasmic TP expression and p53 but this was not

observed with stromal TP expression. The association with p53 has been noted previously [203] suggesting that TP expression might be modulated by p53. Interestingly TP expression did not appear to correlate with vascularity or CA-9. This is in direct contrast to a study in lung cancer [191], where there was significant co-expression of CA9 with TP and a higher MVD. Recently hypoxia has been identified as a microenvironment factor, which can up-regulate TP expression and enzyme activity in breast carcinoma cells [216]. However, other studies have produced contrasting data, with increased expression of TP associated with high blood velocity as measured by Doppler imaging, which is presumably a measure of vascularity and therefore high oxygen tension [217].

The lack of correlation in this current series between TP and CD34 is also interesting as TP has been found to be correlated with MVD in many tumour sites including, gastric [218, 219], cervical [220, 221], endometrial [222], breast [223], renal cell [224] and colorectal carcinomas [165, 174, 225]. Interestingly in a study [226] of endometrial carcinomas only stromal TP correlated with increased MVD and not the tumour staining.

Cyclin A correlated with p53 expression, which may purely be due to the cell-cycle dependence of each protein. There was also a positive correlation between cyclin A and increasing expression of CA-9, suggesting increasing levels of hypoxia with increased tumour proliferation, however this association was not seen with Ki67. In contrast previous reports have [227] demonstrated that CA9 correlated with Ki-67 expression, suggesting a growth advantage in CA9 positive areas.

### **3.6.3 Survival outcome**

In this series of anal carcinomas there was a significant improvement in RFS, OS and CSS for those patients who received combined modality treatment. One of the most significant factors for predicting survival was T stage, however this effect was most prominent in the RT alone treatment arm, T4 tumours in particular had a very poor survival outcome if treated by radiation alone. However, all T stages had similar survivals in the CMT arm and a benefit for CMT even in T1 and T2 tumours was demonstrated.

A series from the Princess Margaret Hospital also showed that tumour size did not appear to be significant in those patients who received combined modality treatment [228]. Salmon and colleagues [229] found that tumour size was significantly related to survival, in which radiation therapy alone was the primary treatment, this fits with the current study. This finding has also been supported in multivariate analyses in other anal carcinoma series [230-232]. In a series of 118 patients treated by external beam and brachytherapy there was an increase in local failure with T stage (T1, 11%; T2, 24%; T3, 45% and T4, 43%) and a corresponding decrease in 5-year survival [233].

In contrast to T stage, the reported effect of positive lymph nodes on survival is less clear. In this current series increasing N stage was associated with both a poorer DFS and CSS and this fits with the Intergroup trial [18], which reported a higher colostomy rate, which is an indirect measurement of local failure, in N1 versus N0 patients (28% vs. 13%). The EORTC [17] trial also reported that patients with positive nodes experienced significantly higher local failure and lower survival. However, there was no difference in prognosis between N1 versus N2 versus N3 disease. In the Princess Margaret series [228] patients with negative nodes who received combined modality therapy had a higher 5-year cause-specific survival compared with those with positive nodes (81% vs. 57%). In contrast other studies have [207, 234] reported no significant difference in 5-year colostomy-free and overall survival in patients with node positive compared with node negative disease, using multivariate analyses.

Grade has been shown to be a prognostic factor in some anal carcinoma studies [235], with low grade tumours resulting in 5-year survivals of 75% compared with only 24% for high grade tumours, however these patients did not receive combined modality treatment. In contrast in this series grade did not impact upon survival, which is in agreement with other studies [236]. No association was found between sex of the patient and outcome. However, male gender has been shown to be associated with poorer local control [237]. A retrospective cohort of 1050 patients with anal carcinoma found that females had a more favourable 5 year survival [236]. The EORTC study also [17] identified male sex as an independent poor prognostic factor for DFS and OS on multivariate analysis.

The high proliferation index in this series has been noted previously [136, 138]. However, no significant association was found with either of the survival endpoints. This lack of impact upon survival has been demonstrated in other studies of anal carcinomas [138] but associations with depth of invasion and lymph node involvement have been shown [139]. A positive correlation between a high Ki-67 index and poor prognosis however, has been reported for upper urinary tract carcinomas [238], astrocytomas [239] and hepatocellular carcinomas [240]. However, inconclusive or contradictory results have been reported for other tumour types, namely oesophageal squamous cell carcinomas [241, 242], gastric carcinomas [243, 244], and lung carcinomas [245, 246].

In this series 78% stained positive for p53, similar to other studies [123, 128]. Alterations in p53 protein function may result from either mutations in its gene or sequestration by other cellular proteins such as the E6 viral oncoprotein of the HPV virus [247]. HPV E6 protein has a direct effect on p53 in the basal layers of the anal epithelium, allowing the continuous proliferation of the host cells and increasing their risk of mutation [248]. Several studies have looked at the association of p53 and HPV. The product of HPV E6 and E7 genes is able to inactivate the p53 and pRb proteins. P53 nuclear accumulation has been found to be associated with the presence of HPV without an effect on clinical outcome [129]. However, in other studies p53 was not found to be associated with HPV and coexpression of p53 and the HPV E6 oncoprotein was uncommon [123, 124, 128]. Mullerat et al [127] studied samples from 70 patients with anal warts, low grade anal intraepithelial neoplasia (AIN), high grade anal intraepithelial neoplasia and anal squamous cell carcinoma. Both the expression of Ki67 and p53 increased significantly and gradually as the lesions became dysplastic and invasive. The main increase in p53 expression was as the lesions progressed from anal warts to low grade AIN. A similar expression of p53 and Ki67 has been reported to increase with the level of dysplasia in pre-invasive anal lesions [249, 250]. The average S-phase fraction has been demonstrated to be significantly higher in HPV positive versus HPV negative lesions with a higher PCNA index [136]. Expression of p53 protein in anal cancer is thus influenced not only by alterations in the p53 gene alone, but may also be dependent on HPV status and this may explain the high levels of expression in anal carcinomas.

In this current series an increased percentage of p53 staining was found to be an independent factor, on multivariate analysis, for a poorer CSS ( $p=0.01$ ) and of borderline significance for a poorer RFS, irrespective of the treatment arm. In a similar smaller study of [125] 49 patients, p53 expression also predicted for a poorer DFS. Other studies however, have been contradictory [124]. The RTOG study [18], of 64 patient found there was a trend for patients whose tumours over-expressed p53 to have an inferior outcome however stage was the only predictive factor in multivariate analysis. A further small study of 18 patients also concluded that p53 had no prognostic impact and the authors concluded that p53 gene overexpression may confer a more aggressive growth pattern, but does not impact upon prognosis [251].

In anal carcinoma there is little data on the potential role of angiogenic markers. A study of CD31 [168], a platelet endothelial cell adhesion molecule, like CD34, showed no correlation with neoplastic relapse but there was a significant correlation with the depth of tumour invasion, supporting the concept that tumour growth is angiogenesis-dependent. Increasing MVD has also been demonstrated to correlate with increasing grade of intra-epithelial anal neoplasia [252], suggesting angiogenesis is a pre-malignant event [253, 254]. The significant finding in this study was that decreasing CD34 was significantly associated with a poorer RFS, in multivariate analysis and CSS in univariate analysis. This was prominent in the RT alone arm but not in those patients who received combined modality treatment. A similar finding has been reported in rectal carcinomas receiving 5FU-based chemoradiation [108], where a low micro-vessel density was prognostic for increased tumour recurrence. It has been suggested that higher vessel densities may facilitate higher concentrations of chemotherapeutic drug reaching the tumour and that radiation may increase vessel leakage also resulting in increased delivery of the chemotherapy to the tumour.

In this series TP expression in the tumour correlated with a poorer RFS overall. However in the CMT group, this survival difference was not seen and the patients with tumours expressing high levels of TP expression had a similar outcome to those that were negative or with low levels of TP expression. The role of TP is complicated by the fact that staining can occur within the tumour stroma, nucleus and cytoplasm. It has been suggested that stromal staining is most representative of TP's angiogenic activity promoting microvessel growth supported by the observation of a correlation



with increased MVD [210, 255]. These observations are in contrast to the present study. It has also been proposed that a nuclear location might indicate a role of regulating thymidine levels for DNA synthesis, while its cytoplasmic location might be required to regulate other enzymes such as TS and thymidine kinase [256].

As a consequence of its involvement in angiogenesis, TP has been identified as a poor prognostic factor in several tumour sites. In a report of patients with gastric carcinoma, [257] TP positive tumours had a significantly worse prognosis. Another study demonstrated the promotion of angiogenesis and a significantly higher rate of incidence of hepatic metastasis in patients with TP positive gastric carcinomas [258]. However, in carcinomas of the breast [259] and bladder [211], no correlation between TP expression and recurrence free survival, or overall survival was found.

In this current series, TP may well be acting as a poor prognostic factor as a direct result of its role as an angiogenic factor; although this does not explain the lack of influence seen in the CMT arm. The CMT is a more effective treatment and the prognostic effect may be lost in the same way as the effect of T stage, alternatively there could be an interaction between the 5FU and TP. The latter has already been suggested in other studies [260, 261] where TP was found to potentiate commonly used cytotoxic drugs such as 5-Fluorouracil (5FU) and other thymidylate synthase inhibitors as well as methotrexate. Fox et al [259, 262] has previously shown that TP is upregulated in breast cancer epithelium by 20 fold and also showed that TP expression was associated with treatment response in a series of 328 breast carcinomas treated with adjuvant CMF (cyclophosphamide, 5FU and methotrexate) [263]. In the latter study univariate analysis revealed an association between TP and RFS but not OS in all patients and the node positive sub-group, no influence of TP was observed in the node negative group. Patients treated with CMF showed a significant increase in RFS and OS in TP positive tumours; this was not evident in patients not treated with CMF.

These results suggest that patients with TP-positive tumours have a significant survival benefit when treated with CMF. The advantage of TP positivity may be due to TP enhancing tumour sensitivity to cytotoxic therapy, particularly methotrexate and 5FU. Since the enzymic pathway of TP is reversible, the activation of 5FU may

be enhanced with increased conversion to 5FdUMP [261, 264], an active metabolite of 5FU.

In a reported study of 98 patients, 51 of whom received combined modality treatment, lack of bcl-2 expression was associated with lower local control and overall survival, this remained significant on multivariate analysis [146]. Patients with positive bcl-2 and negative p53 tumours had a significantly higher 5-year LC compared to patients with negative bcl-2 and positive p53 (93% vs. 53%). In contrast in this present series no association between outcome and bcl-2 was demonstrated.

Data on the prognostic value of cyclin D1 is rare, although overexpression has been reported to be an adverse factor in hypopharyngeal carcinomas [265] and carcinomas of the anterior tongue [149] predicting for an increased lymph node status and poor DFS and OS. In this study cyclin D1 did not predict for outcome.

CA9 is a marker of hypoxia and although expression of CA9 has been noted in other tumour sites; cervical [266], oesophageal [267], colorectal [227] and lung cancer [268], its clinical and prognostic role in human malignancies is unclear. Hypoxia in other carcinomas especially squamous cell carcinomas, has been shown to be important in predicting response to treatment and influencing clinical outcome [162]. The role of hypoxia in anal squamous cell carcinomas and how it may influence response to chemoradiation is largely unexplored. In a study of lung cancer [191], multivariate analysis showed that CA9 was a significant prognostic factor independent of angiogenesis. In this series of anal squamous cell carcinomas CA9 stained positively in 80% of cases. However, unlike in other squamous carcinomas, there was no significant correlation with either of the survival indices or response to chemoradiation on multivariate analyses.

There are few reports on IHC studies to evaluate the tumour expression of DPD [269, 270] or the relationship between clinical outcome and DPD expression in humans [269, 271-273]. Therefore, the clinical implication of intra-tumoral DPD levels is not clear. Recently in non-small cell lung carcinoma a poorer survival was demonstrated in those patients whose tumours had a high expression of DPD [271]. However, in this series, DPD expression did not correlate with survival. The lack of correlation

with DPD in the CMT arm was an interesting finding because a recent study in bladder carcinomas has demonstrated that the activity of DPD was inversely correlated with sensitivity to 5FU [212] and a low DPD intratumoral gene expression in colorectal tumours was associated with tumour response to 5FU [274]. This has also been demonstrated in colorectal cancer cell lines [275].

#### **3.6.4 Treatment response**

This current series has demonstrated that response to treatment is an important prognostic factor. Outcome is significantly improved with a CPR or >50% response to treatment. Similar findings were reported [233] in 118 patients where the initial response to radiotherapy was also evaluated after a 6-8 week interval. Response was shown to influence locoregional control as much as survival, and appeared to be the main prognostic factor, this group also found that a cut-off of <50% response to be a good selection criteria to assess poor responders.

Patients in this study who did not achieve a greater than 50% response and went on to salvage surgery did not do well, with the majority of patients surviving no longer than 18 months. However, this contrasts with the results of a small review of 21 patients, who underwent an abdominoperineal resection for persistent disease following RT/CMT who had a 72% 3 year survival [276]. An explanation for the disparity in survival for this group of patients is that in the current series a significant proportion of the poorer responders were of higher T stage and in a retrospective study [277] of T4 tumours, salvage surgery did not influence outcome, due to higher rates of distant recurrence. This same retrospective study also found T4 tumours benefited more from chemoradiation as found in this current study. Leichman et al [206] noted no recurrence of tumour in 38/45 patients who were tumour free after chemoradiation with 5FU and MMC. All those with persistent disease developed recurrence at distant sites. This stresses the importance of achieving eradication of disease with CMT.

For all the anal carcinoma patients taken together, increasing T stage, N stage and p53 predicted for a poorer response to treatment. The same factors predicted response in the CMT treatment arm. In the RT alone arm associations between response and

several other factors also became apparent. Increasing bcl-2 and decreasing cyclin A predicted for a poorer response to radiation alone.

The association of response with tumour T stage has been noted previously in anal carcinomas [234]. In a study of [278] 35 patients treated with chemoradiation a 100% complete response rate was reported in T1/2 tumours and 60% in T3 tumours. The Intergroup and EORTC trials [17, 18] also reported a higher complete response rate by tumour size. Unlike in this current series these results were based on univariate analyses. The association of response with N stage is not as well documented and previous reported studies have found no difference between node positive and node negative tumours [278].

A higher expression of cyclin A predicted for an improved response to irradiation. Willett et al [279] reported a marked pathological downstaging after preoperative irradiation of rectal adenocarcinomas with higher Ki-67 indices compared with tumours with low indices and an improved survival has been observed in patients with high grade lymphomas receiving chemotherapy, whose Ki-67 indices were greater than 80% [280].

P53 in this series also predicted for a poorer response to treatment overall and in the chemoradiation arm. In a previously reported study of rectal carcinomas p53 expression correlated inversely with response to chemoradiation and a CPR, and directly with an increased likelihood of residual cancer in the lymph nodes of the surgical specimens [107]. A correlation between p53 immunohistochemical staining and a poor response to chemotherapy has also been observed in patients with lung and ovarian cancers [102, 103]. Recent studies have confirmed that induction of apoptosis by the agents commonly used in cancer treatment is highly dependent on normal p53 function [281-284].

Bcl-2 inhibits the induction of apoptosis from a wide variety of stimuli including radiation and chemotherapy [144]. Overexpression potentially promotes resistance to both treatment modalities. However, in this group of patients overexpression of bcl-2 only predicted for a poorer response in the RT alone arm.

This series demonstrates that certain molecular markers have been identified which may predict outcome and response to treatment in patients with squamous cell carcinoma of the anus. In particular this group of patients were randomised to one of two treatments, with differing outcomes. This enabled a more detailed investigation of the role of these molecular markers in response to different treatment modalities. This is also one of the first studies to comprehensively investigate the role of biological molecular markers and their degree of expression in this carcinoma site.

## **Chapter 4:**

# **Molecular Markers in Rectal Carcinoma**

## Chapter 4

### **Defining potential predictive and prognostic molecular markers in locally advanced rectal carcinoma**

#### **4.1 Aims**

- Determine the overall survival and relapse-free survival for a cohort of locally advanced rectal cancer patients treated with 5FU chemoradiation
- Determine the importance of downstaging and an R0 resection
- Define predictive molecular markers for the effectiveness of chemoradiation in rectal carcinoma
- Identify new prognostic indices that will predict the outcome in this group of rectal carcinomas
- Identify whether molecular markers are up-regulated or down-regulated by chemoradiation

#### **4.2 Introduction**

The Mount Vernon Hospital Cancer Centre serves a population of 1.8 million. A prospective database has collected information on all the locally advanced rectal cancer patients, who have been treated with 5FU-based chemoradiation prior to surgery. Tumours were defined as rectal if the lower limit was located within 12cm of the anal verge on rigid sigmoidoscopy. Information on all these patients was retrieved from the database.

#### **4.3 Patients**

Between January 1995 and December 2002, patients with locally advanced rectal cancer were identified. Initial staging by digital rectal examination (DRE), computerised tomography (CT) and more recently magnetic resonance imaging (MRI), defined these patients as locally advanced or unresectable (stage T3/4) rectal cancers. A total of 155 patients had been treated with 5FU and low dose folinic acid in combination with radiotherapy. All patients had confirmed adenocarcinoma of the

rectum and they were considered unresectable by the referring surgeons. There was no evidence of distant metastases at the commencement of treatment.

#### **4.4 Treatment**

The patients received chemotherapy with bolus low dose folinic acid ( $20\text{mg/m}^2$ ) and 5FU ( $350\text{mg/m}^2$  over a 60 minute infusion) on days 1-5 and 29-33 of radiotherapy. Pelvic radiotherapy delivered a dose in the majority of cases of 45Gy in 25 fractions over 33 days, to a planned volume.

Surgery was performed 6-8 weeks after finishing chemoradiation. After histopathological examination of the resected specimen, patients with a positive circumferential resection margin ( $\leq 1\text{mm}$ ), extra-nodal deposits or Dukes C histology went on to receive post-operative adjuvant 5FU-based chemotherapy.

Resection margins were defined using UICC criteria:

- R0: No microscopic residual tumour
- R1: Microscopic residual tumour
- R2: Macroscopic residual tumour

#### **4.5 Handling of specimens**

Formalin fixed, paraffin embedded tissue blocks from both the original diagnostic biopsy and surgical specimens were requested for all of the 155 patients. A total of 60 biopsy and 33 surgical tissue blocks were received. Samples were cut into  $4\mu\text{m}$  sections using a rotary microtome. Sections were then mounted onto poly-L-Lysine coated microscope slides and dried overnight at  $37^\circ\text{C}$ .

#### **4.6 Molecular markers**

A panel of 6 molecular markers were examined. These markers were chosen as indices of proliferation, differentiation and cell cycle control. The contribution of thymidylate synthase (TS) and thymidine phosphorylase (TP) were also studied in the context of the 5FU chemotherapy. All of these markers have been implicated as prognostic or predictive indices in rectal carcinoma, in previous studies.



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Proliferation:	Cyclin A & EGFR
Recruitment:	P53
Redistribution:	Cyclin A
Apoptosis:	P53 and bcl2
Tumour structure:	Grade
5FU:	TS and TP
Differentiation:	EGFR

The panel of molecular markers were examined to provide a basic profile of the tumours. This profile was analysed in conjunction with known prognostic pathological and clinical indices, which were recorded as part of the prospective database.

#### **4.6.1 Marker Staining**

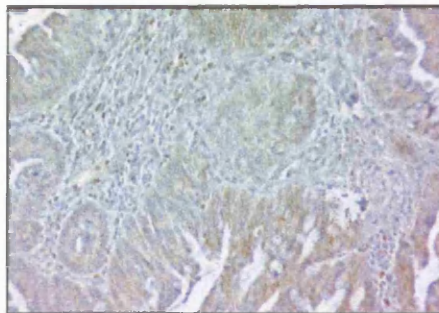
Figure 4.1 demonstrates the staining patterns for each marker in the rectal carcinoma specimens. The common distributions of staining for each marker were discussed in the methods and materials chapter.

#### **4.7 Statistical analysis**

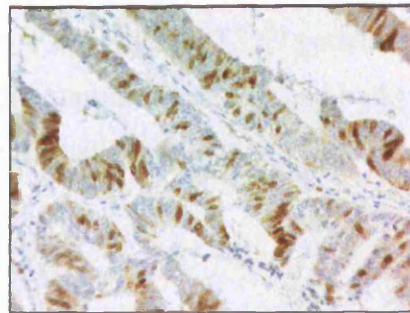
The main endpoint was relapse-free survival (RFS), with secondary endpoints of overall survival (OS) and response to chemoradiation.

A univariate analysis was performed using Kaplan-Meier curves for the association between each of the markers and known pathological prognostic indices for RFS, CSS and overall survival (OS). Comparison between the different marker scores with the endpoints was compared by the logrank test and associations between markers were analysed using the Spearmans Rank correlation coefficient. A multivariate analysis was then undertaken using the Cox proportional hazards model. This method has been described in Chapter 3.

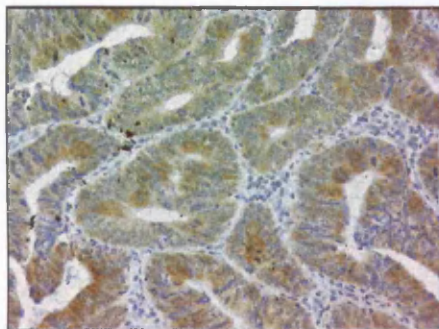
For the analysis the statistics package used was JMP version 5.0 (Statistical Discovery Software, SAS Institute) and SPSS for windows release 11.5.0 (Statistical package for Social Sciences).



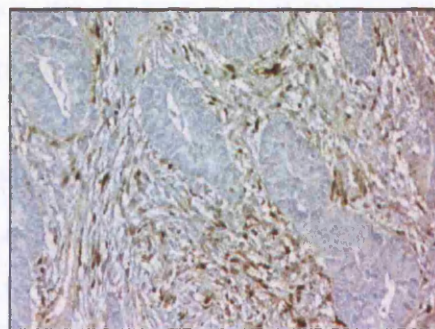
Bcl-2



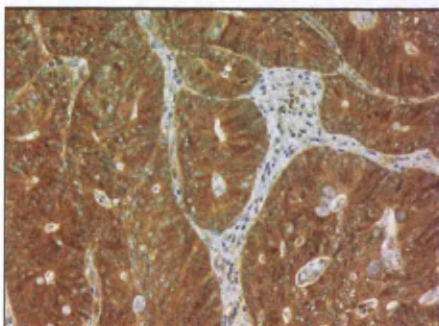
Cyclin A



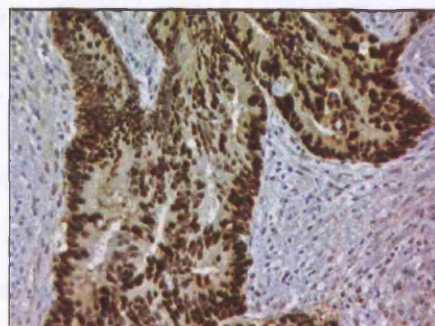
EGFR



TP



TS



P53

**Figure 4.1** Examples of molecular marker expression patterns in rectal cancer

## **4.8 Results**

### **4.8.1 Patient characteristics**

The median age for the 155 patients was 67 years (range 21-85) with a male:female ratio 113:42 (73%:27%). The T stages were: T2:2 patients, T3:59 patients and T4:94 patients. In the case of the two T2 patients the tumours were low and bulky. Curative surgery was performed in 122 patients. The median follow-up was 25 months and in this time 67 (43%) patients died.

### **4.8.2 Survival**

#### **4.8.2.1 Relapse-free survival**

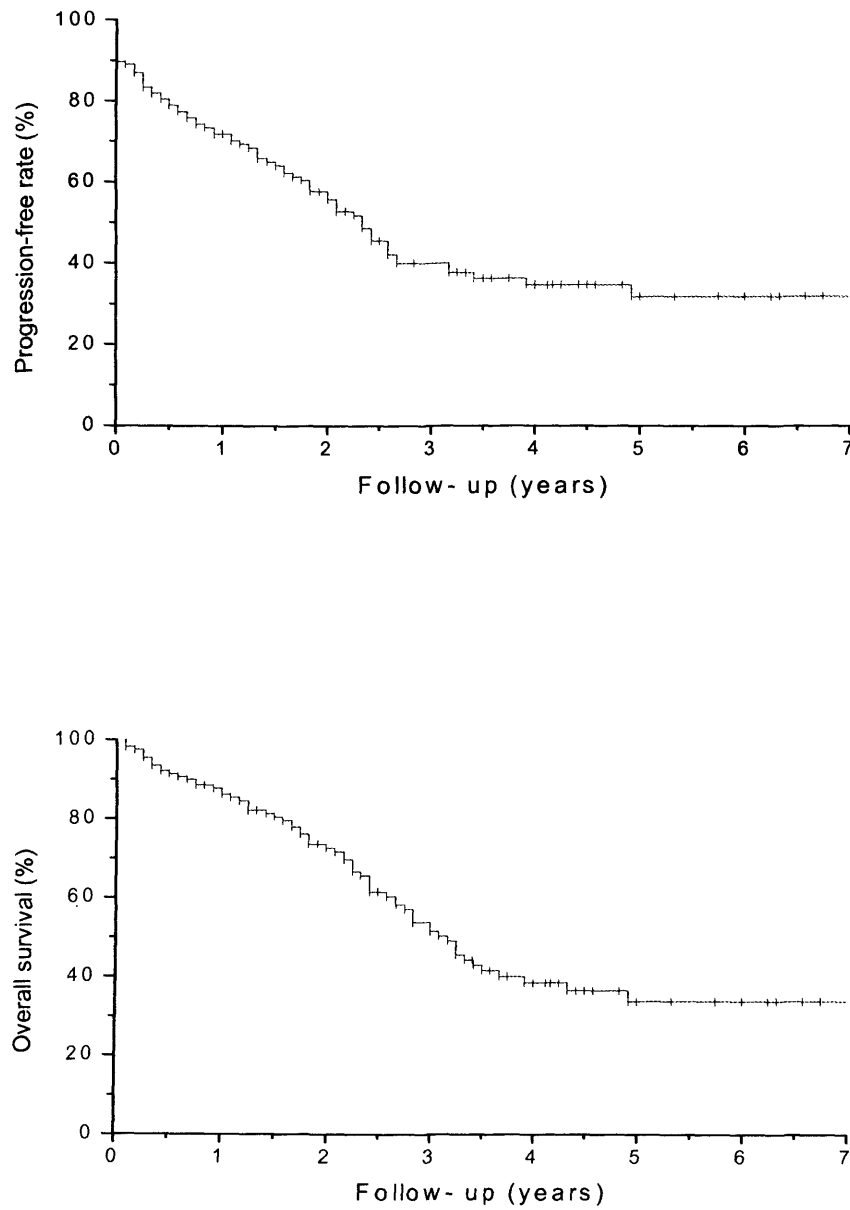
The median RFS was 28 months (2.33 years) and 31% were disease-free at 5 years of follow-up, see Figure 4.2. The median time to local recurrence (LR) was 22 months and 19 months for the median time to distant metastases (DM).

The median overall survival (OS) was 37 months (3.08 years) and 34% were alive at 5 years of follow-up. From these results it is clear that this group of patients have a poor OS with a high risk of both LR and DM.

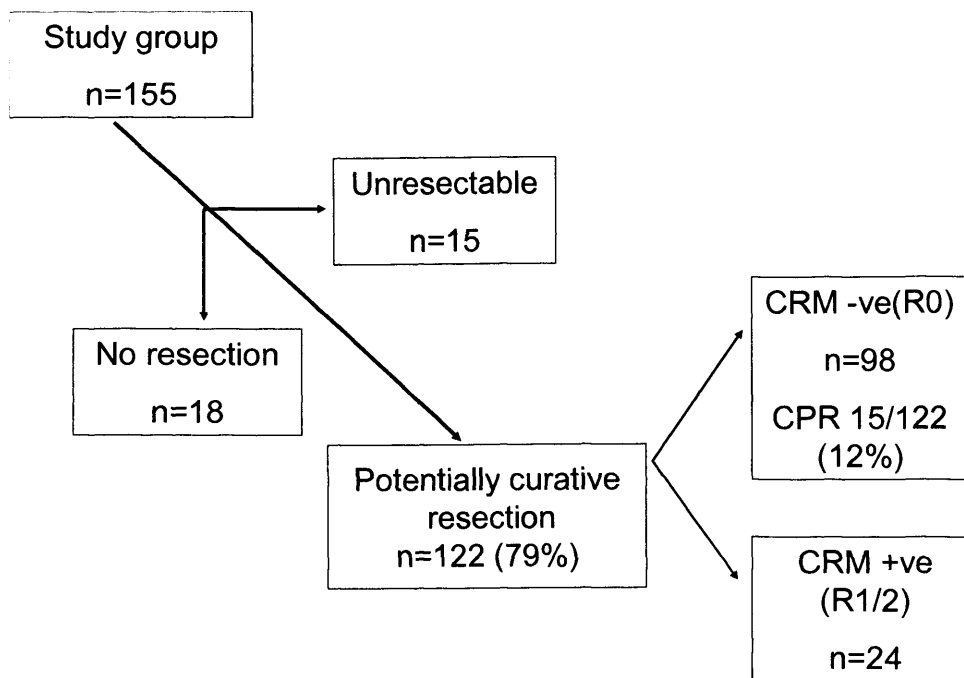
#### **4.8.2.2 DFS and OS for an R0 resection**

All of the 155 patients were deemed unresectable and therefore likely to have an R1/2 resection margin, by the referring surgeons. Of the 155 patients who underwent chemoradiation 122 had undergone surgery with curative intent, see Figure 4.3.

After completing chemoradiation 15 patients were still unable to undergo a curative resection and underwent palliative surgery. No resection had been performed on a further 18 patients for various reasons, 4 patients died shortly after completing CRT or during surgery, 3 had been lost to follow-up, 5 patients refused surgery who had had a complete pathological response (CPR), 2 patients who had prior cardiac disease were felt unsuitable to undergo surgery and 5 patients were still awaiting their surgical resection.



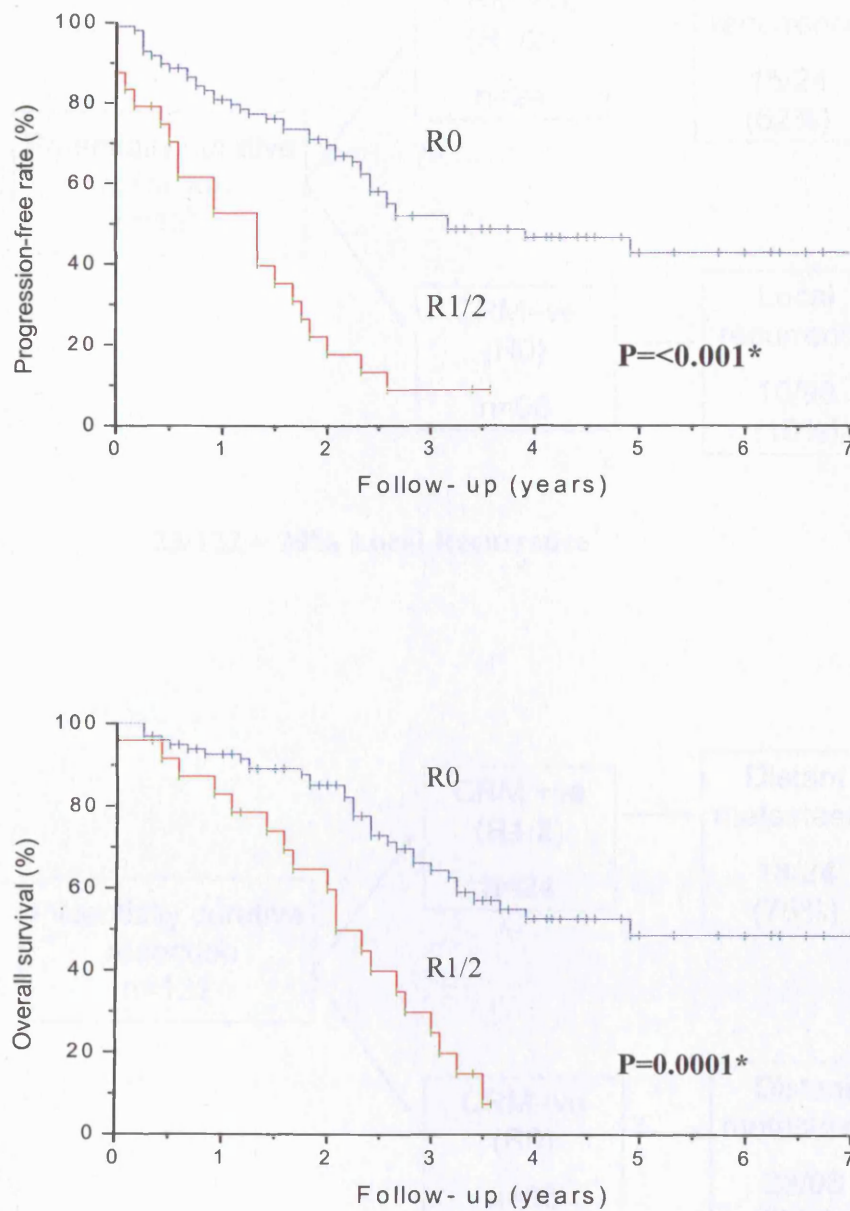
**Figure 4.2** Progression-free and overall survival in the study patients



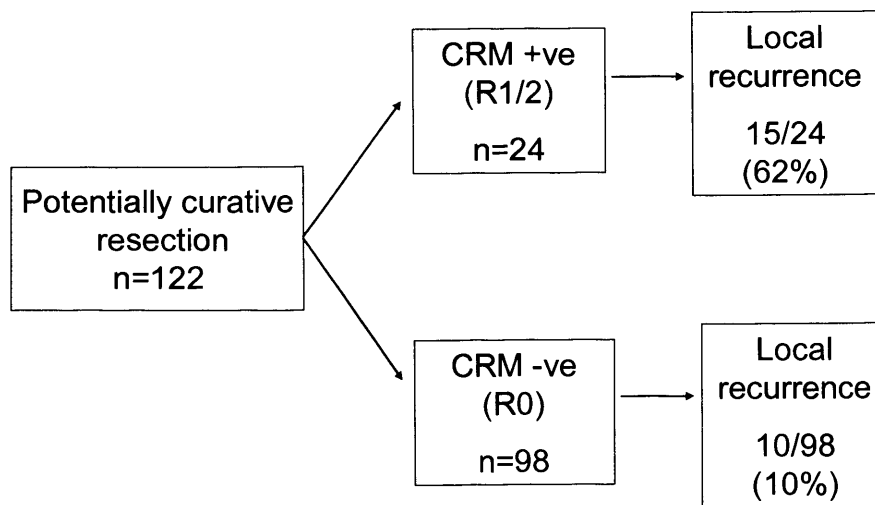
**Figure 4.3** Clinical outcome and management of study group

Of the 122 patients who underwent curative surgery, an R0 resection was achieved in 98/122 cases (80%), with an overall R0 resection rate for the entire group (98/155) of 63%. The importance of achieving an R0 resection in terms of survival can be seen in Figure 4.4 below.

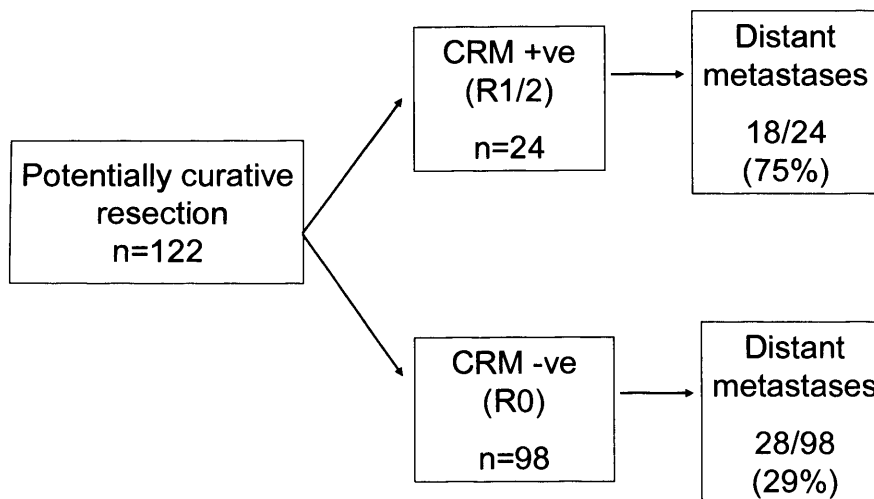
As can be seen in Figures 4.5.A and B, both the LR and DM rates are higher in the R1/2 resection group. This results in a significant difference between the two groups in both DFS and OS. The median DFS in the R0 resection group was 38 months with a 3 year DFS of 52%. This is in contrast to the R1/2 resection group where the median DFS is 16 months with a 3 year DFS of only 9%. The difference between the two groups is strongly significant log rank  $p < 0.001$ .



**Figure 4.4** Importance of achieving a clear circumferential margin and progression-free (upper) and overall survival (lower)



**23/122 = 20% Local Recurrence**



**38/122 = 37% Distant metastases**

**Figures 4.5.A and B** Incidence of local recurrence (upper) and distant metastases (lower) in relation to the circumferential margin

The median OS was 59 months in the R0 resection group and the 3 year OS was 64%. In the R1/2 resection group the median OS was 25 months with a 3 year OS of 25%. The difference in OS between the two groups was strongly significant log rank  $p=0.0001$ .

It is clearly important from these figures to achieve an R0 resection in this group of patients. With this chemoradiation regimen 62% of patients previously deemed unresectable had a curative R0 resection. Pathological downstaging to pT0/1/2 occurred in 28% of cases and 15/122 (12%) achieved a complete pathological response (CPR), however at least a further 5 patients who refused surgery also appeared to have had a complete response.

#### 4.8.2.3 Response to chemoradiation

For the purpose of this analysis response was divided into two groups, those whose tumours had been downstaged to pT0/1/2 by chemoradiation and those whose tumours had not.

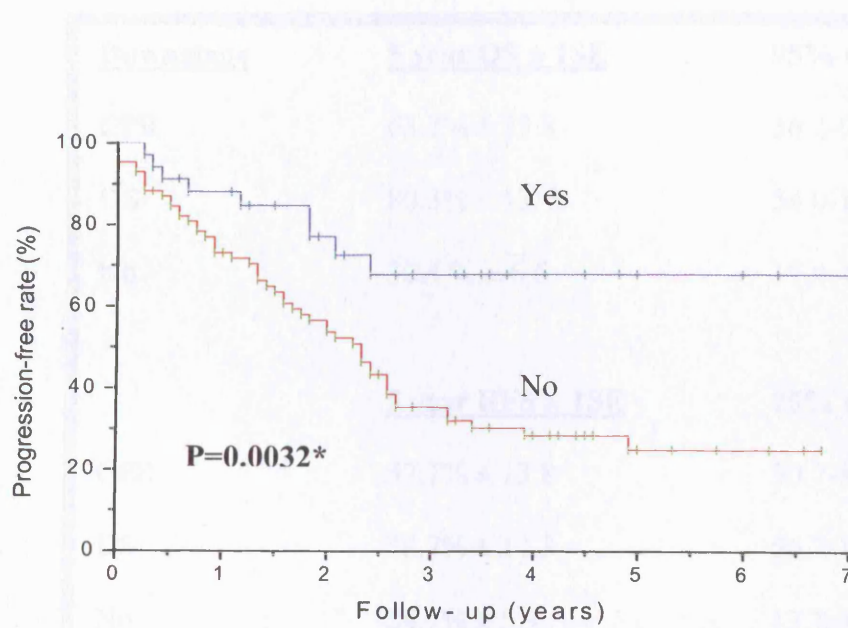
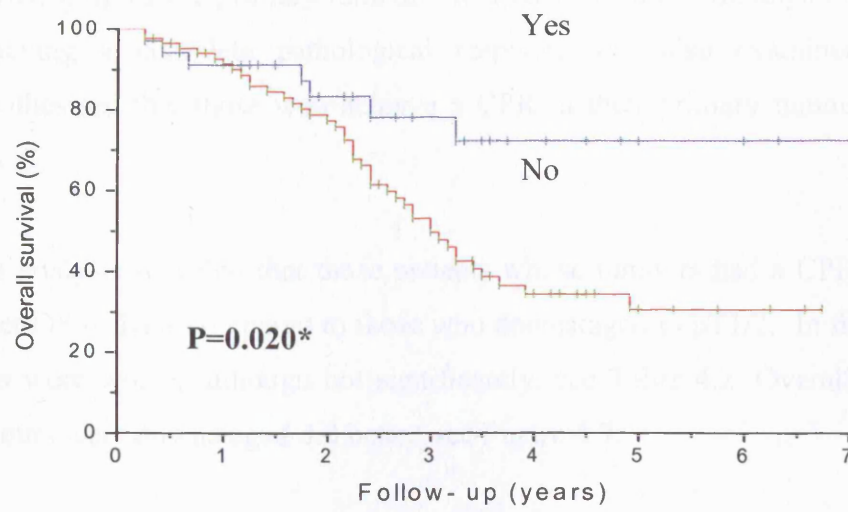
##### 4.8.2.3.1 Survival analysis

RFS and OS were analysed for the different response groups. This showed there was a significant difference at 5 years follow-up for both parameters. See Table 4.1. Figure 4.6 clearly demonstrates the impact of downstaging on survival.

<b><u>Downstage</u></b>	<b><u>5 year OS <math>\pm</math> 1SE</u></b>	<b><u>95% CI</u></b>
Yes	71.9% $\pm$ 9.6	53.1-90.7
No	30.4% $\pm$ 6.6	17.5-43.3
	<b><u>5 year RFS <math>\pm</math> 1SE</u></b>	<b><u>95% CI</u></b>
Yes	67.6% $\pm$ 9.2	49.6-85.6
No	24.7% $\pm$ 5.9	13.1-36.3

**Table 4.1** Survival outcome in relation to pathological downstaging of the primary rectal tumour





**Figure 4.6** The effect of downstaging on overall and progression-free survival

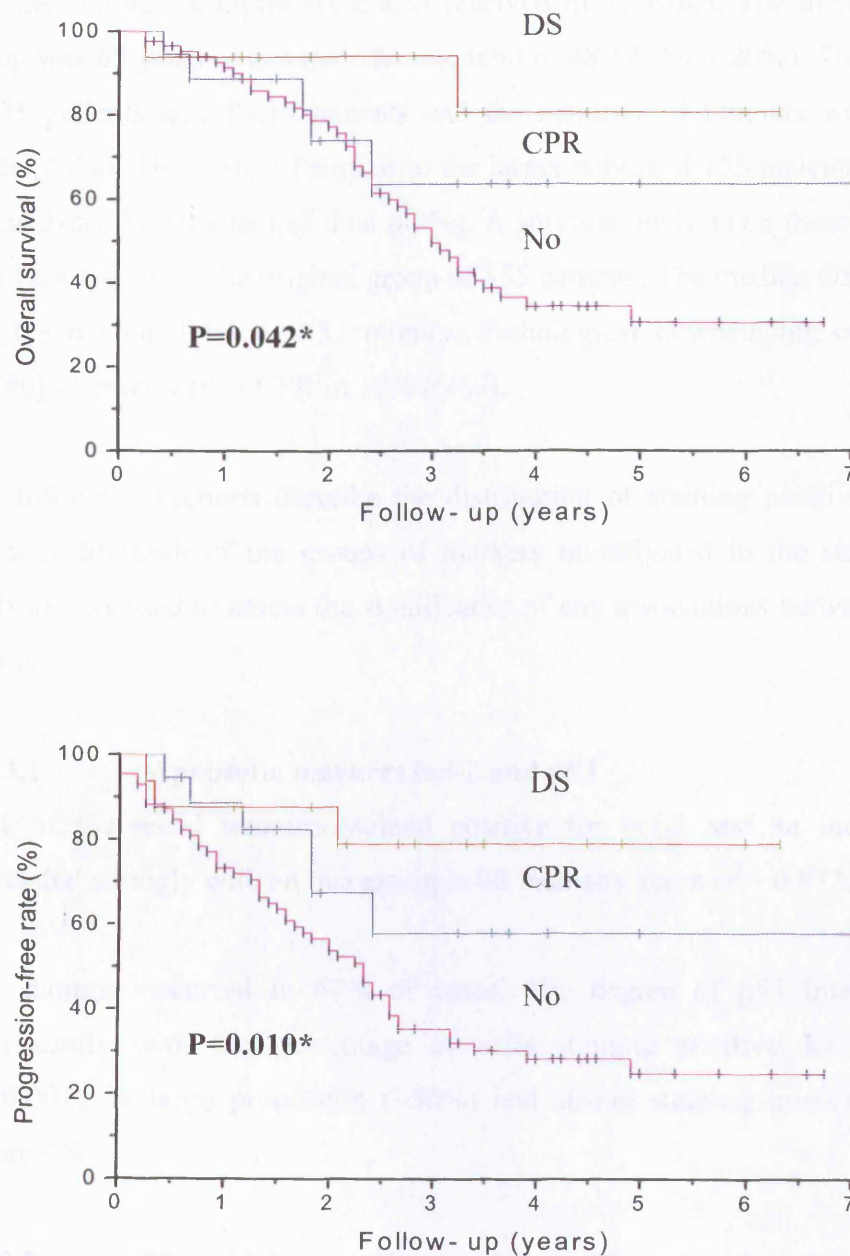
#### 4.8.2.3.2 Importance of a complete pathological response

The results show that it is important in terms of both RFS and OS to achieve downstaging of the primary tumour. As a result of these findings the importance of achieving a complete pathological response was also examined. It has been hypothesised that those who achieve a CPR in their primary tumour will do better overall.

This analysis revealed that those patients whose tumours had a CPR did not have a better OS or RFS compared to those who downstaged to pT1/2. In fact their survival rates were worse; although not significantly, see Table 4.2. Overall patients whose tumours were downstaged did better, see Figure 4.7.

<b><u>Downstage</u></b>	<b><u>5 year OS <math>\pm</math> 1SE</u></b>	<b><u>95% CI</u></b>
CPR	63.2% $\pm$ 13.8	36.2-90.2
DS	80.3% $\pm$ 13.4	54.0-106.6
No	30.4 % $\pm$ 6.6	17.4-43.4
	<b><u>5 year RFS <math>\pm</math> 1SE</u></b>	<b><u>95% CI</u></b>
CPR	57.7% $\pm$ 13.8	30.7-84.7
DS	78.7% $\pm$ 11.2	56.7-100.7
No	24.7% $\pm$ 5.9	13.1-36.3

**Table 4. 2** The effect of response on survival



**Figure 4.7** The influence of downstaging or complete pathological response on overall and progression-free survival

### **4.8.3 Biopsy marker frequencies**

From the original 155 patients, 60 biopsy samples were received. From these 60 patients, surgical samples were also received in 33 cases. The median age for this group was 68 years with a male:female ratio of 48:12 (80%:20%). The T stages were: T3:21 patients and T4:39 patients and the majority of tumours were of moderate grade (65%). These are all similar to the larger cohort of 155 patients. At the time of the analysis 26 patients had died (43%). A survival analysis on these 60 patients was also very similar to the original group of 155 patients. The median OS was 40 months and the median DFS was 31 months. Pathological downstaging occurred in 28% (16/60) of cases with a CPR in 10% (6/60).

The following sections describe the distribution of staining positivity and staining intensity for each of the groups of markers investigated in the study. Correlation analysis was used to assess the significance of any associations between intensity and positivity.

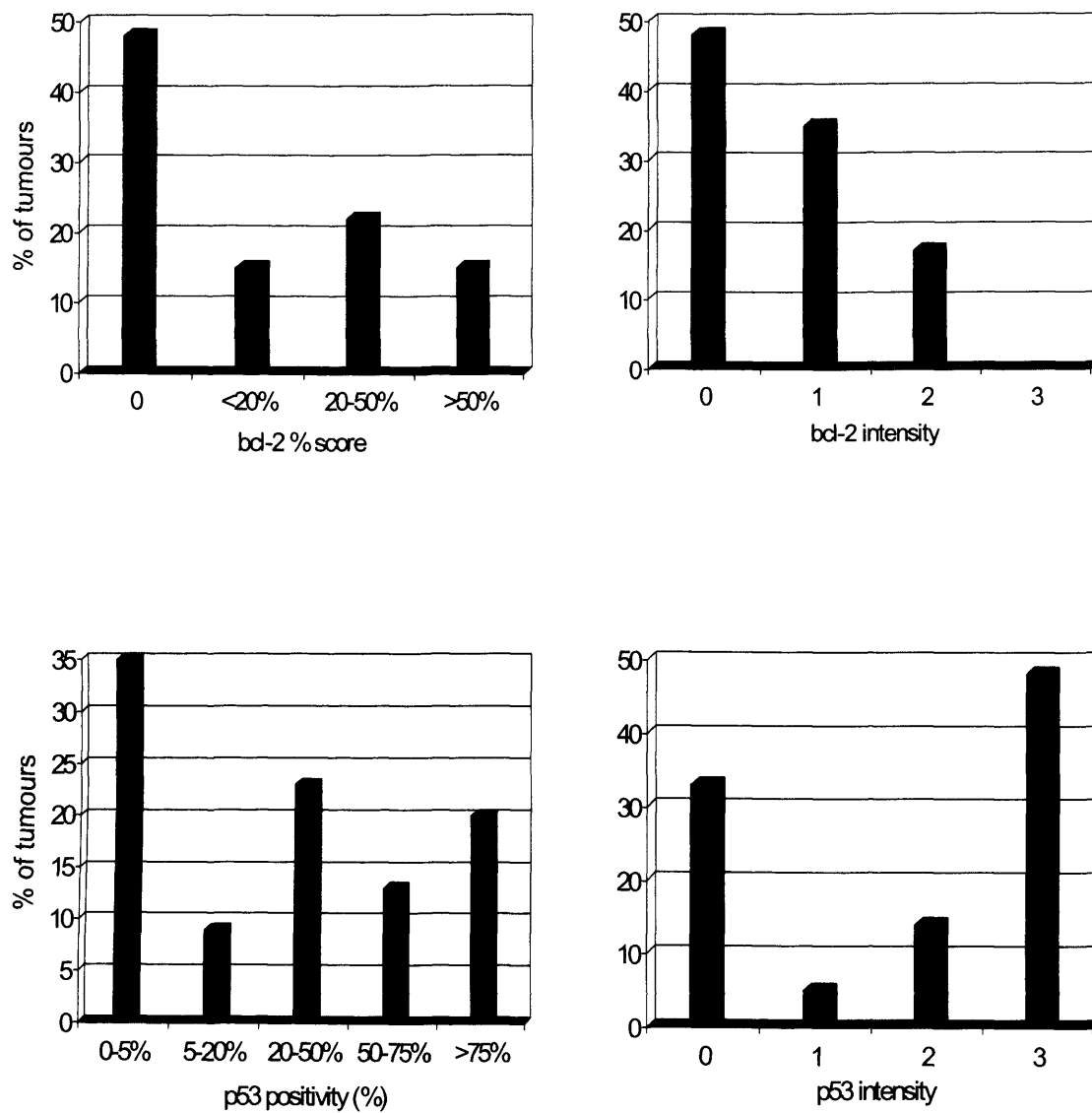
#### **4.8.3.1 Apoptotic markers bcl-2 and p53**

52% of the rectal tumours stained positive for bcl-2 and an increasing bcl-2% correlated strongly with an increasing bcl-2 intensity score ( $r^2 = 0.877$ ,  $p < 0.001$ ).

P53 staining occurred in 67% of cases. The degree of p53 intensity correlated significantly with the percentage of cells staining positive for P53 ( $r^2 = 0.775$ ,  $p < 0.001$ ). A large proportion (~50%) had strong staining intensity for p53, see Figure 4.8.

#### **4.8.3.2 Thymidylate synthase and thymidine phosphorylase**

Staining for TS in the rectal samples was different from that in the anal cancer samples. Although most of the rectal biopsies stained positive for TS (95%), there was quite a variation in the percentage of cells stained and so this was also scored for the rectal tumours. The majority of the TS staining was of weak to moderate intensity. TS intensity correlated strongly with TS percentage ( $r^2 = 0.592$ ,  $p < 0.001$ ). See Figure 4.9.



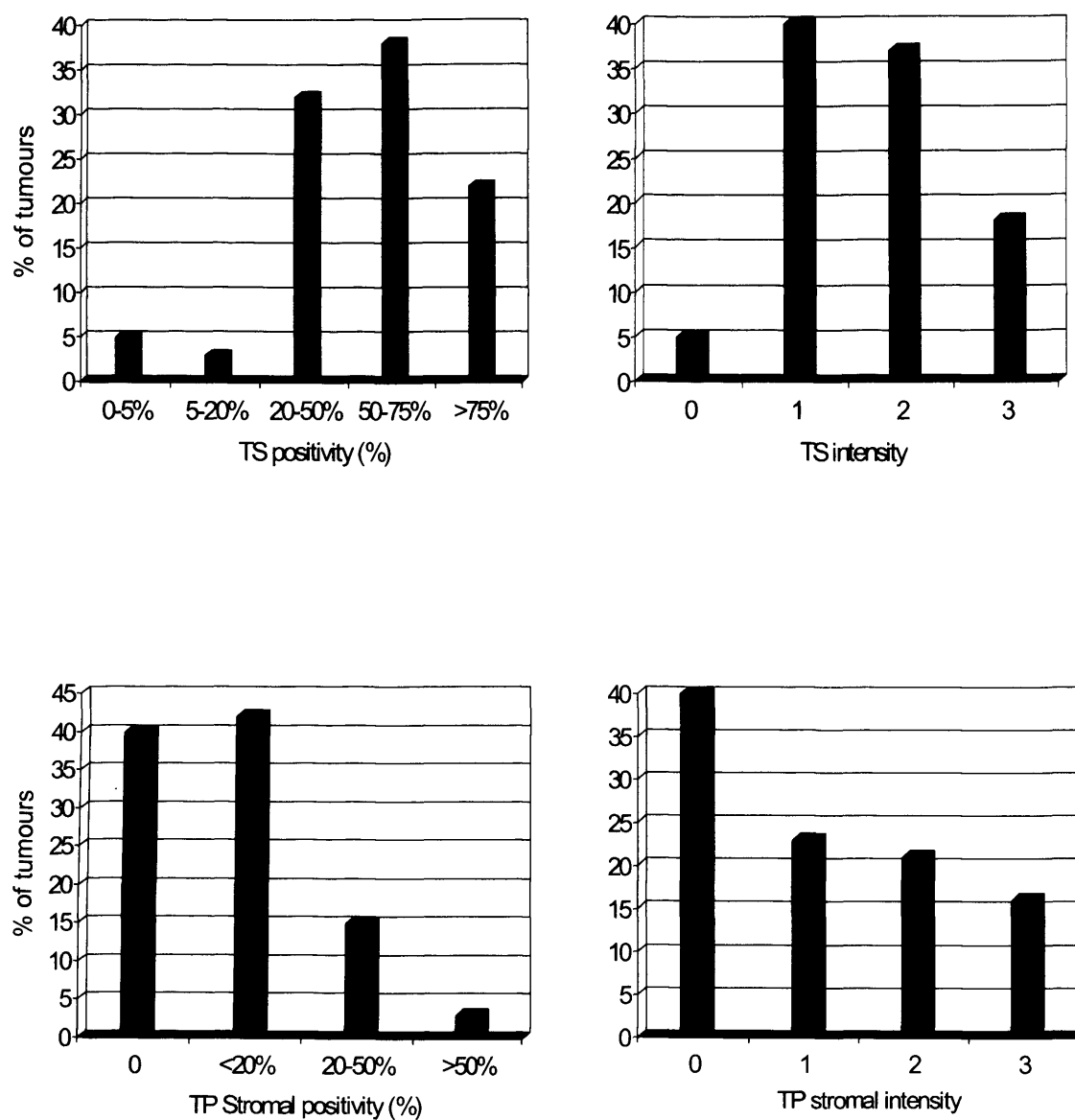
**Figure 4.8** The distribution of apoptosis-related proteins

In contrast to the anal cancer biopsies only 60% of cases stained positive for TP and staining appeared to only occur in the tumour stroma. TP stromal percentage correlated strongly with intensity ( $r^2 = 0.898$ ,  $p < 0.001$ ). See Figure 4.9.

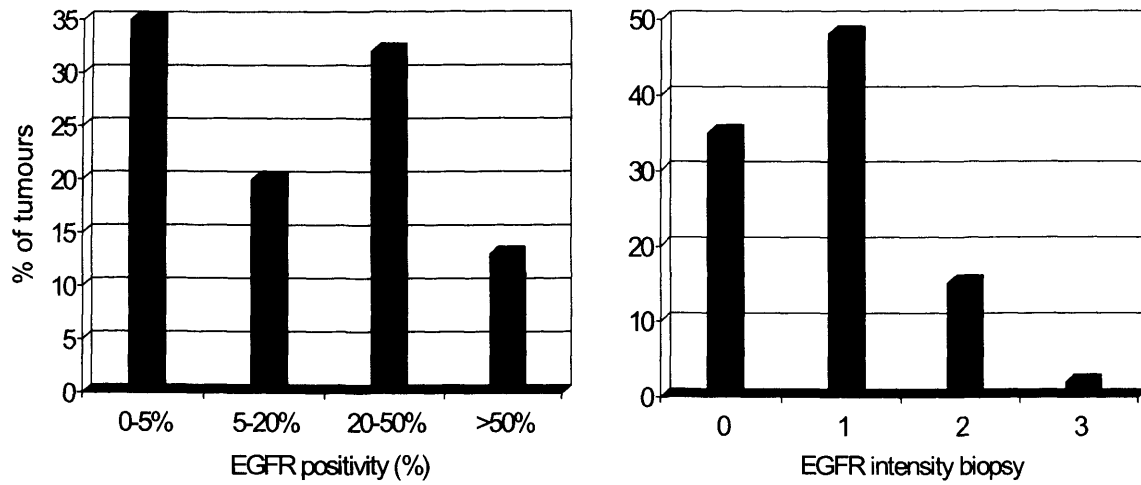
#### **4.8.3.3 EGFR and Cyclin A**

EGFR staining can be cytoplasmic and membranous. In the rectal cancer specimens the staining was only present in the cytoplasm. To check that the antibody was working several other types of tumour were stained and membranous staining did occur in the squamous cell carcinoma controls. There was a strong positive correlation between the EGFR intensity score and the percentage of cells stained ( $r^2 = 0.843$ ,  $p < 0.001$ ). See Figure 4.10.

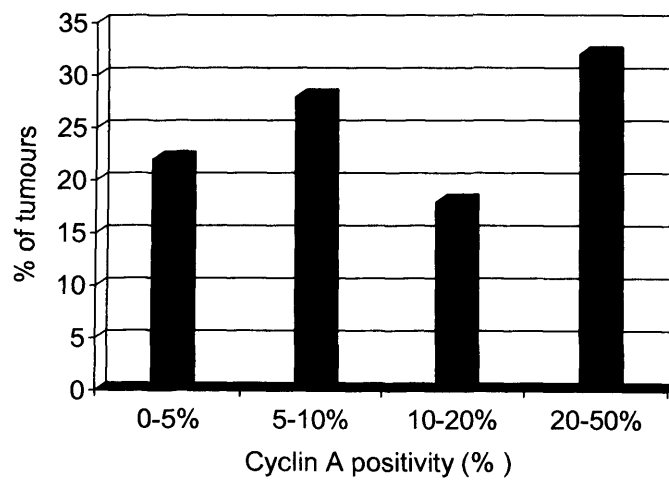
Over 78% of cases stained for cyclin A. The majority of samples, >95% scored 1 on intensity so the percentage of cells stained was the scoring used. See Figure 4.11.



**Figure 4.9.** The distribution of TS and TP



**Figure 4.10** The distribution of EGFR



**Figure 4.11** The distribution of cyclin A



#### **4.8.4 Surgical marker frequencies**

For 33 of the patients in the study, surgical sample specimens were also received. The aim of analysing these was to see if the expression of the markers changed following chemoradiation and whether this influences their value as potential prognostic and predictive markers. The following analysis shows the changes in intensity of expression and percentage of cells staining for each of the markers pre and post surgery.

##### **4.8.4.1 Markers of 5FU response: TP and TS**

Figures 4.12 and 4.13 demonstrate that there is a no significant difference in TP or TS expression between the biopsy and surgical specimens.

##### **4.8.4.2 EGFR expression**

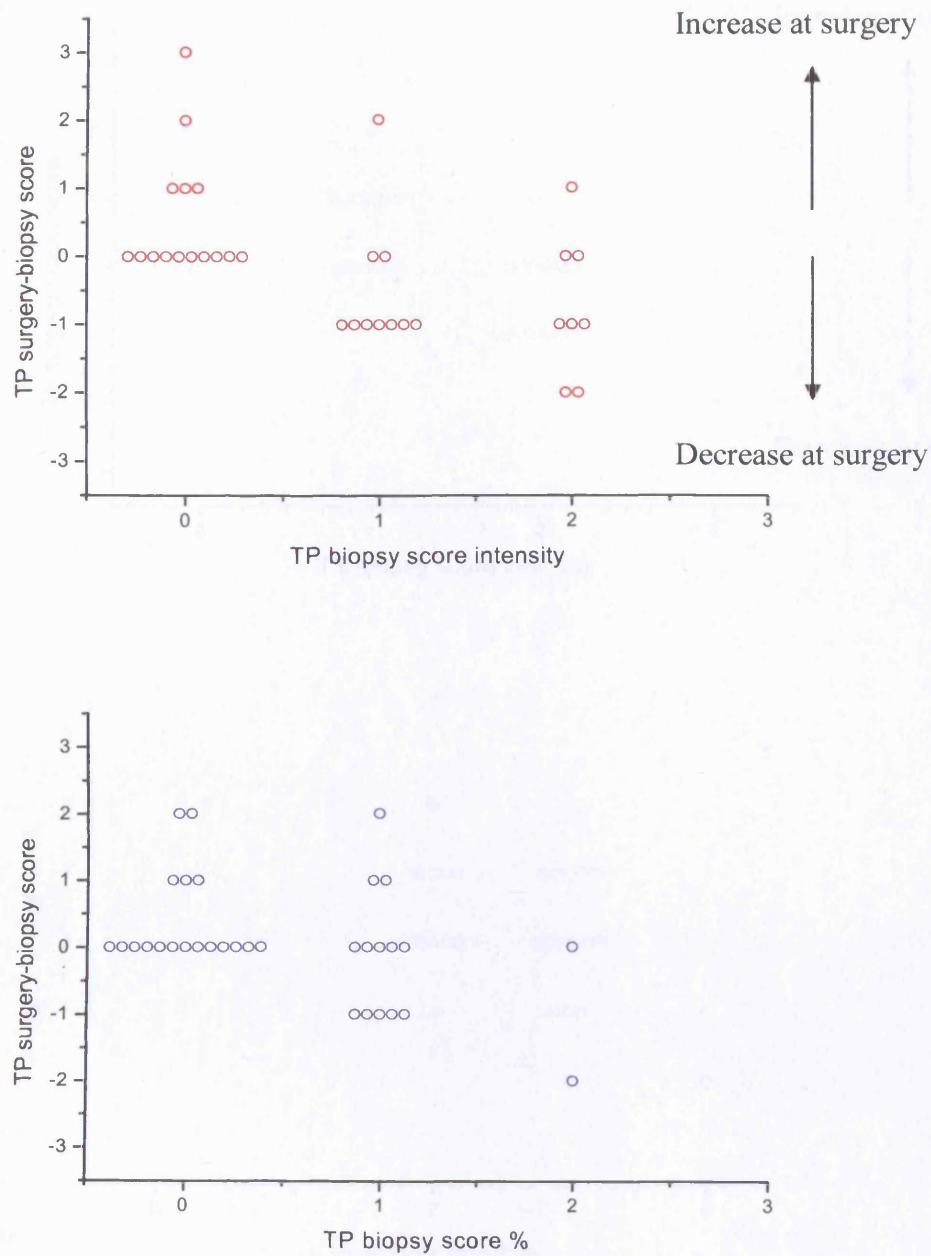
Figure 4.14 shows that there is a trend for an increased EGFR expression in the surgical specimen post chemoradiation. Suggesting an upregulation in expression.

##### **4.8.4.3 Proliferation marker: Cyclin A**

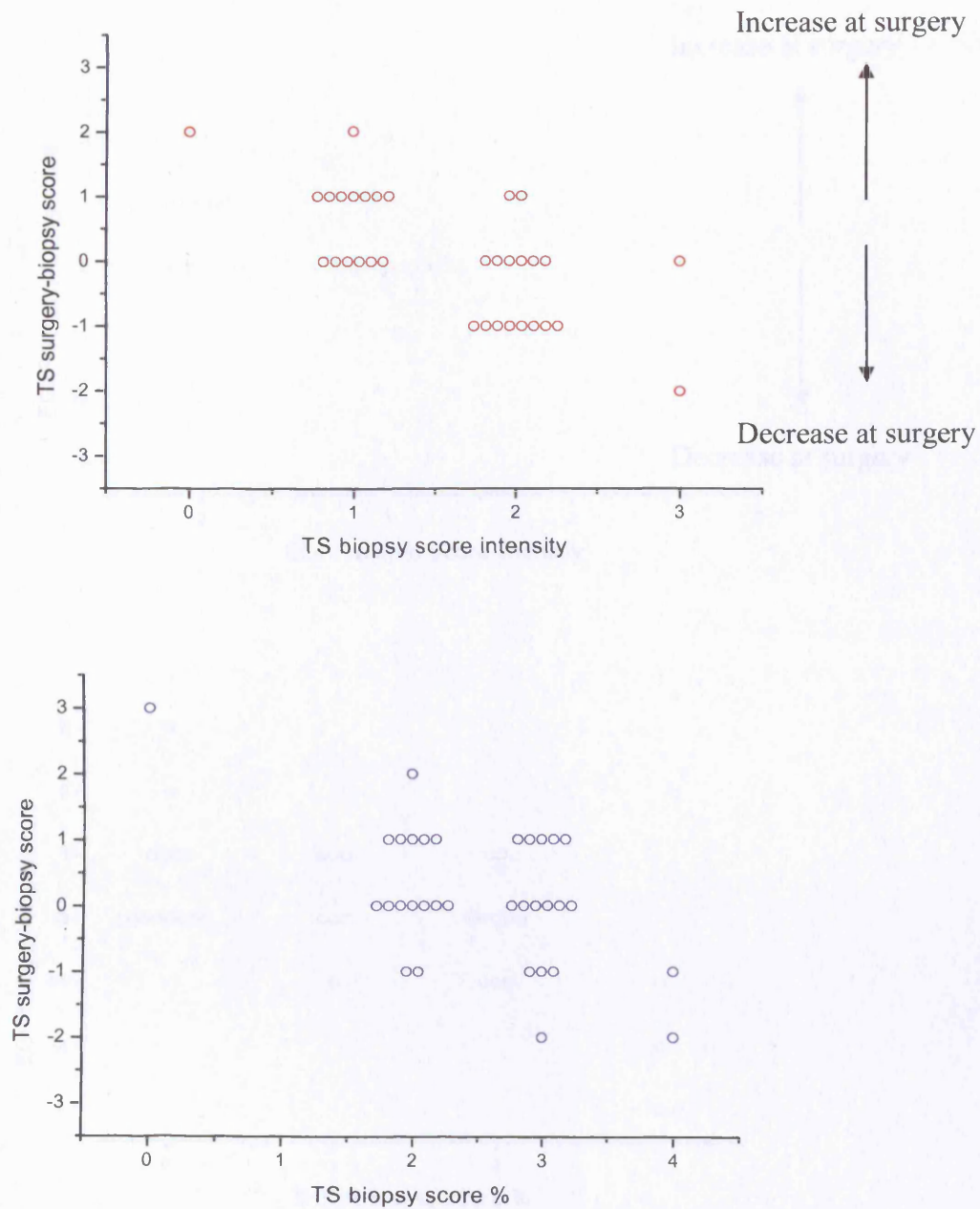
Figure 4.15 demonstrates that there is an overall decrease in the expression of cyclin A following chemoradiation, suggesting an overall downregulation in tumour proliferation. This has been confirmed in other series.

##### **4.8.4.4 Apoptosis markers: bcl-2 and p53**

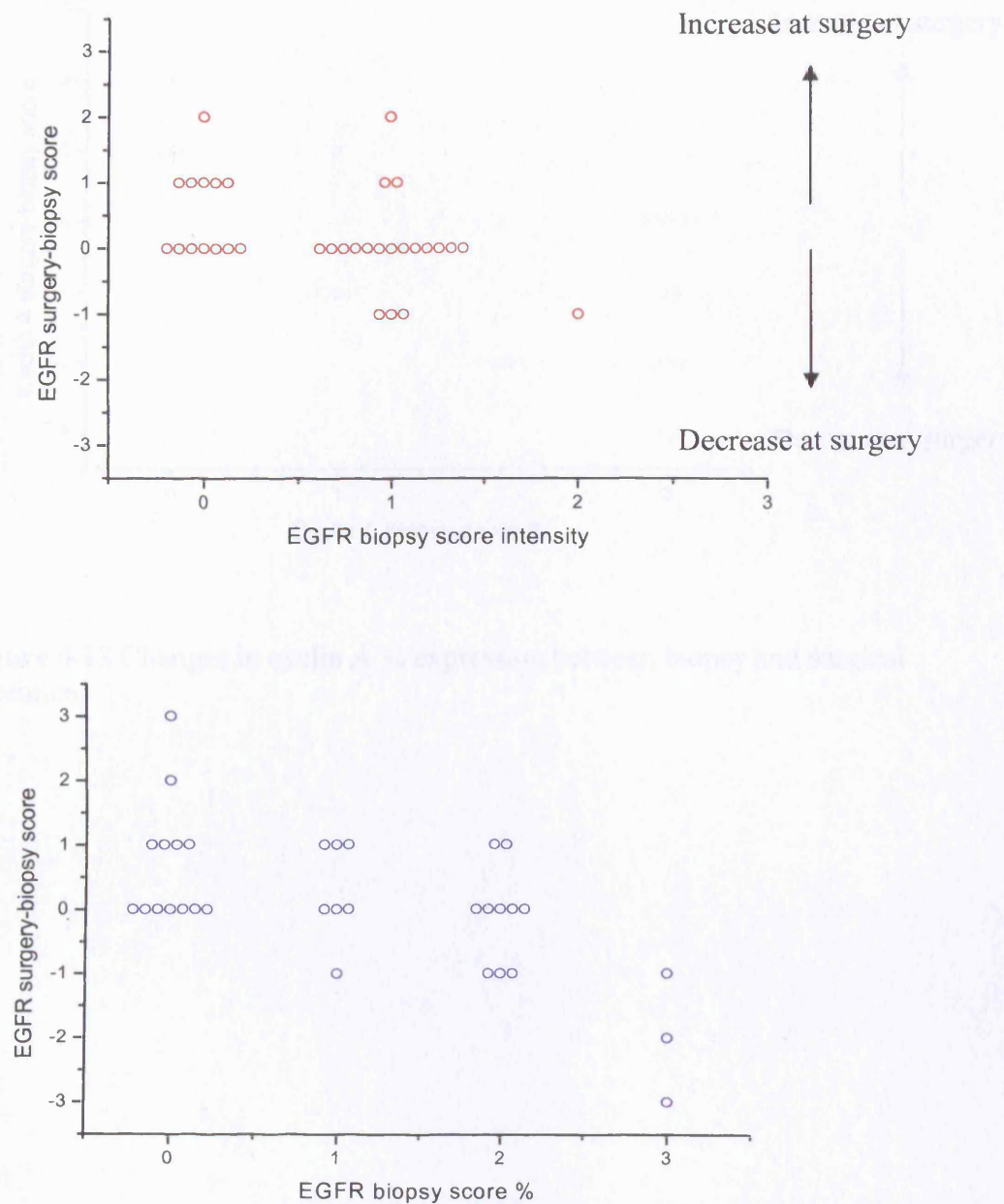
Figure 4.16 shows there appears to be an increase in bcl-2 expression following chemoradiation but there is no similar change in p53 which remains unchanged in expression, see Figure 4.17.



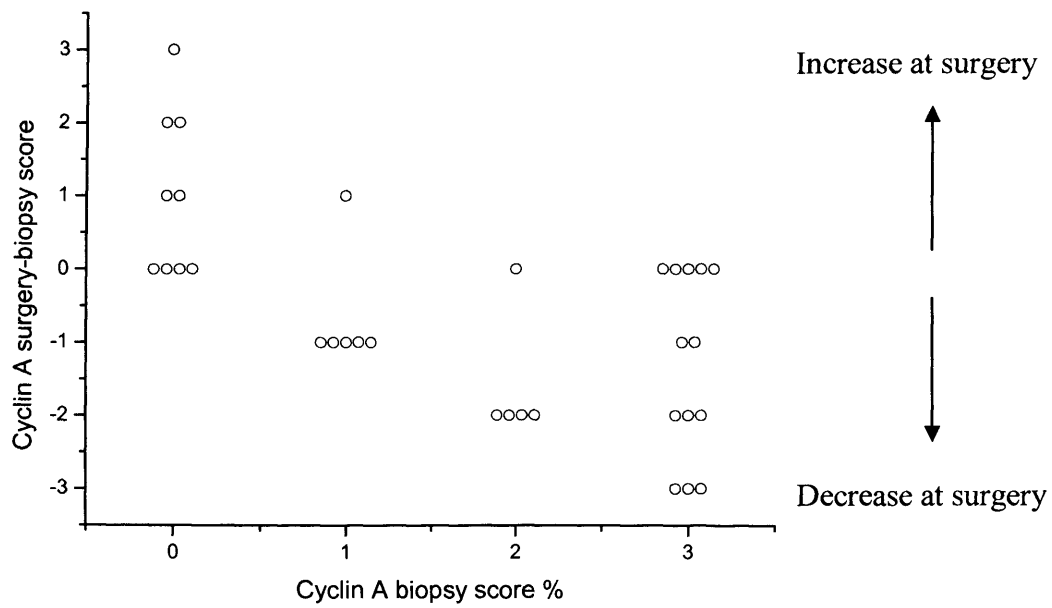
**Figure 4.12** Changes in TP intensity (upper) and % (lower) expression between biopsy and surgical specimens



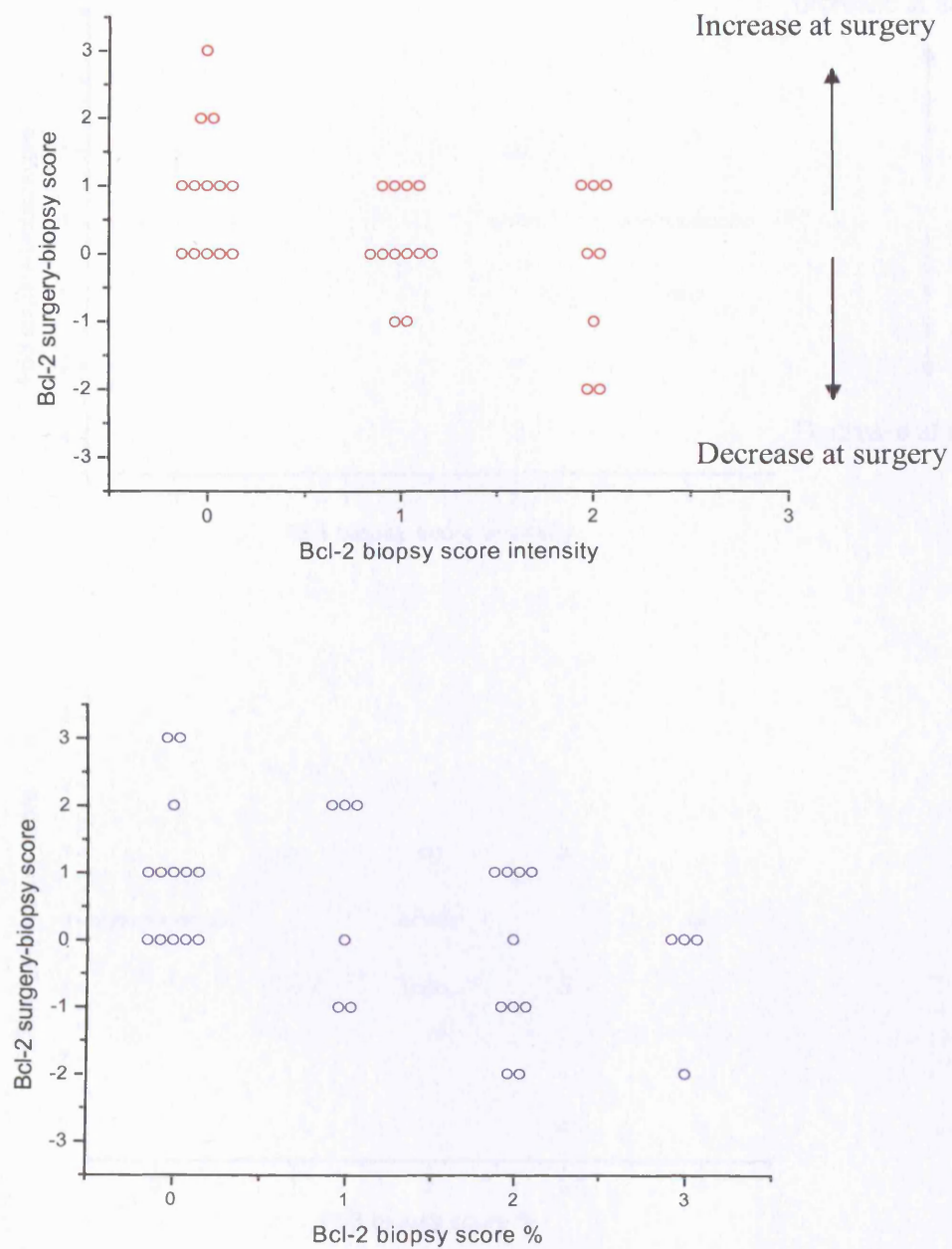
**Figure 4.13** Changes in TS intensity (upper) and % (lower) expression between biopsy and surgical specimens



**Figure 4.14** Changes in EGFR intensity (upper) and % (lower) expression between biopsy and surgical specimens



**Figure 4.15** Changes in cyclin A % expression between biopsy and surgical specimens



**Figure 4.16** Changes in bcl-2 intensity (upper) and % (lower) expression between biopsy and surgical specimens





#### **4.8.5 Correlation between biopsy and surgical markers and clinicopathological variables**

As part of the Mount Vernon database several clinicopathological variables were recorded. These included the sex of the patient, T and N stage, tumour grade, patient age and degree of tumour downstaging. Table 4.3, shows the significant correlations between these clinicopathological features and the biopsy and surgical markers.

In the biopsy specimens there were several correlations with T and N stage, however the rectal tumours were all locally advanced T3 or T4 and more than two thirds were T4 tumours. So any positive associations with staging may not be reliable, because there is little variability in these two clinical characteristics. T stage was positively correlated with increasing cyclin A% indicating that proliferation increases with the higher the T stage. Interestingly there were negative correlations between N stage and bcl2 intensity and TS and TP percentage.

Grade correlated with cyclin A, a marker of proliferation. But there was no association with bcl2, p53 or TS, as seen with the anal samples.

There were strong correlations between increased tumour downstaging and a higher TP intensity and percentage. A higher cyclin A percentage was also associated with improved tumour downstaging. This indicates that in rectal cancer, the more highly proliferating tumours with an increased expression of stromal TP respond best to chemoradiation.

Age correlated with a number of biopsy markers. These included increasing bcl2 intensity and percentage, increasing cyclin A percentage and an increasing percentage of cells staining for TS.

Compared to the biopsy markers there were few significant correlations with clinicopathological variables for the surgical marker expression. Those correlations, which were significant, were not the same as those seen with the biopsy samples.



<u>Clinicopath</u>	<u>By variable</u>	<u>Spearman's Rho</u>	<u>P value</u>
<b><u>Biopsy</u></b>			
T stage	Cyc A%	0.2674	0.041
N stage	Bcl2 Int	-0.263	0.048
N stage	TS% & Int	-.330/-.348	0.012/0.007
N stage	TP%	-0.305	0.019
Age	TS%	0.270	0.039
Age	Cyc A%	0.280	0.032
Age	Bcl2 int	0.392	0.002
Age	Bcl2%	0.427	0.0008
Grade	Cyc A%	0.288	0.049
Downstaging	TP int	-0.339	0.008
Downstaging	TP%	-0.331	0.010
Downstaging	Cyc A%	-0.287	0.028
<b><u>Surgery</u></b>			
N stage	EGFR%	0.369	0.049
N stage	P53% & Int	0.417/0.348	0.011/0.038
Grade	Bcl2 Int	-0.338	0.044
Grade	Bcl2%	-0.318	0.058
Downstaging	P53 Int	0.335	0.046

**Table 4.3** Correlation between biopsy and surgical markers with the clinical characteristics

Bcl2 was negatively correlated with grade and increasing p53 intensity was associated with a poorer response to chemoradiation, and an increasing N stage. The correlation with bcl2 maybe explained by the difference in expression between the surgical and biopsy specimens, see Figure 4.16; however this correlation was seen in the anal samples. In this analysis surgical EGFR% also correlated positively with increasing N stage.

Unlike in the biopsy samples there was no correlation between any of the surgical markers and the age of the patient. There was also no correlation between the proliferation and angiogenic markers with downstaging.

#### **4.8.6 Correlations between individual biopsy markers**

Several hypotheses were generated to look at the associations between some of the markers and determine what their predominant role is.

##### **4.8.6.1 Markers of 5FU response: TP and TS**

Table 4.4 shows the correlations for TS and TP with the other markers. Unlike in the anal samples TS did not correlate with cyclin A, another marker of proliferation. However it did correlate strongly with TP stromal intensity and percentage and with EGFR intensity and percentage. The correlation with TP and lack of correlation with cyclin A may indicate that in rectal tumours TS may have a more significant role in influencing the effect of 5FU. There is a borderline positive association with p53, which may give support to the hypothesis that in rectal tumours at least p53 may be involved in the modulation of TS [202]. There is a positive correlation with EGFR, a marker of differentiation and proliferation. This marker has been associated with more aggressive tumours in several studies [187].

The physiological role of TP is complex and is involved in DNA metabolism and angiogenesis. In contrast to the staining pattern seen in anal cancer, rectal cancers showed mainly stromal localisation, with no obvious staining within the rectal tumour cells themselves. This staining within the stroma may well be the predominant angiogenic component of TP.

<b><u>TS</u></b>	<b><u>By variable</u></b>	<b><u>Spearman's</u></b>	<b><u>P-value</u></b>
TS Int	TP% & Int	0.407/0.343	p=0.001/0.008
TS%	TP% & Int	0.413/0.413	p=0.001/0.001
TS Int	EGFR % & Int	0.255/0.272	p=0.054/0.039
TS%	EGFR% & Int	0.303/0.351	p=0.021/0.007
TS Int	P53% & Int	0.244/0.245	p=0.067/0.066
<b><u>TP</u></b>	<b><u>By variable</u></b>	<b><u>Spearman's</u></b>	<b><u>P-value</u></b>
TP%	EGFR% & Int	0.339/0.416	p=0.009/0.001
TP Int	EGFR% & Int	0.354/0.423	p=0.006/<0.001
TP%	TS% & Int	0.413/0.407	p=0.001/0.001
TP Int	TS% & Int	0.413/0.343	p=0.001/0.008
TP%	P53%	0.278	p=0.037
TP Int	P53%	0.294/0.174	p=0.027

**Table 4.4** Thymidylate synthase and thymidine phosphorylase correlations

The association with p53 has been noted previously [203]. It has been hypothesized that TP expression might be modulated by p53. The association with TS may well suggest that this is due to the angiogenic component. More DNA synthesis occurring in those tumours with a high expression of TP, this would also explain the association with EGFR. It may also be possible that the positive correlation between the two markers may show a role for influencing the response to 5FU based chemotherapies.

#### 4.8.6.2 Epidermal growth factor receptor

EGFR strongly correlated with a number of markers, see Table 4.5. There were strong positive correlations with TP, TS and P53.

#### 4.8.6.3 P53

It can be seen that p53 has significant positive correlations with TS and TP. This may be explained by the fact that P53 has been implicated in the modulation of expression of both of these proteins. There is also a positive correlation between EGFR intensity and percentage of cells stained with the number of p53 cells staining positively.

<u>EGFR</u>	<u>By variable</u>	<u>Spearman's</u>	<u>P-value</u>
EGFR%	TP% & Int	0.339/0.354	p=0.009/0.006
EGFR Int	TP% & Int	0.416/0.423	p=0.001/<0.001
EGFR%	TS% & Int	0.303/0.256	p=0.021/0.054
EGFR Int	TS% & Int	0.351/0.272	p=0.007/0.039
EGFR%	P53%	0.314	p=0.018
EGFR Int	P53%	0.253	p=0.060

**Table 4.5** EGFR correlations

### 4.8.7 Correlations between individual surgical markers

All the marker variables correlated significantly between their intensity and percentage scores. See appendix G for the full list of correlations. The significant correlations are shown below in Table 4.6. There are clearly fewer significant correlations between the surgical markers than in the biopsy markers.

The correlations between bcl-2 and cyclin A were not seen in the biopsy analysis. There is a significant correlation between an increasing bcl-2 with a decreasing cyclin A expression. There is also a positive correlation between cyclin A and TP.

TS correlates with cyclin A, another marker of proliferation, as seen in the anal samples but not in the rectal biopsy markers. However there is no correlation with TP or p53. The same correlation between TS stromal intensity with EGFR intensity and percentage is seen and there is also a positive correlation between TS and bcl-2.

<u>Variable</u>	<u>By variable</u>	<u>Spearman's</u>	<u>P-value</u>
TS Int	EGFR% & Int	0.373/0.442	p=0.050/0.019
Cyc A%	TP% & Int	0.437/0.491	p=0.013/0.004
Cyc A%	TS%	-0.359	p=0.048
Bcl2%	Cyc A%	-0.457	p=0.005
Bcl2 Int	Cyc A	-0.449	p=0.006
Bcl2%	TS%	0.377	p=0.033

**Table 4.6** Correlations between surgical markers

## **4.8.8 Marker survival analysis**

### **4.8.8.1 Relapse-free survival**

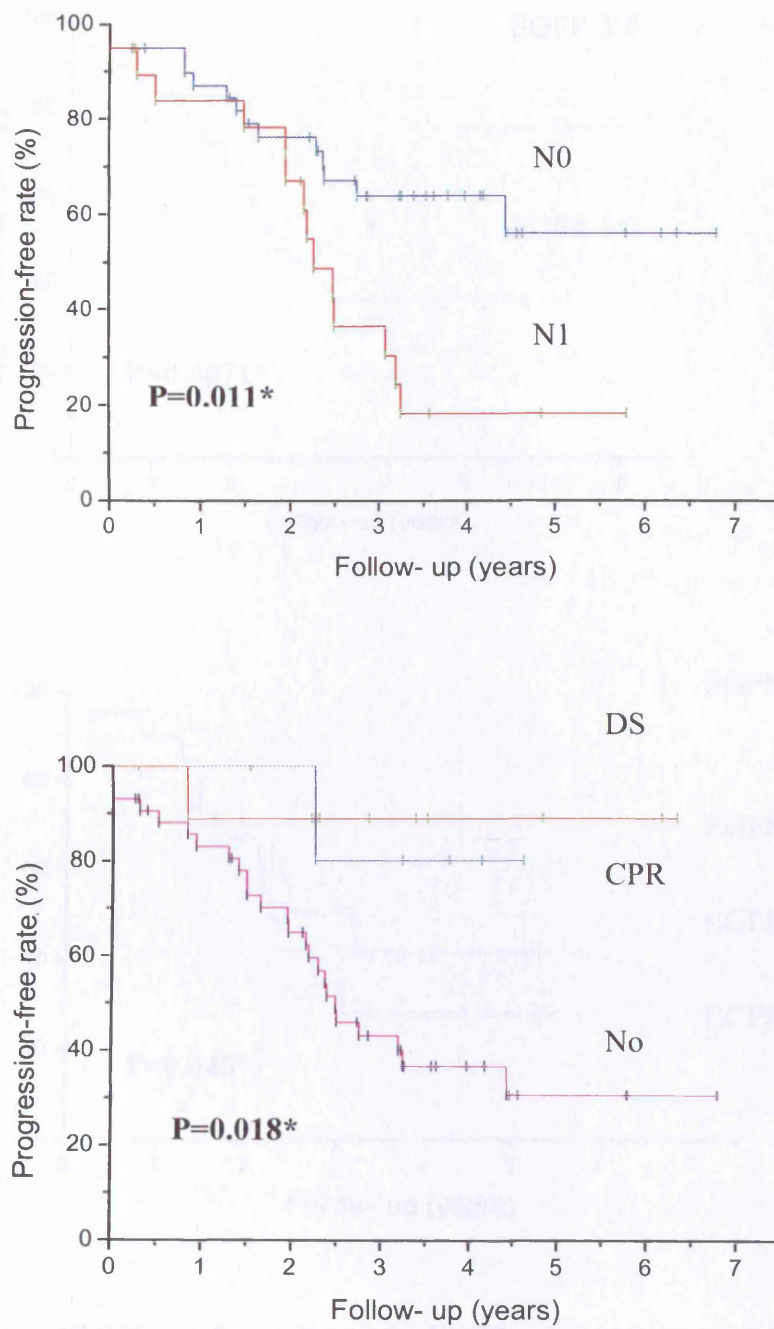
With univariate analysis increasing N stage was statistically significant for a poorer RFS. The lack of significance for T stage is likely in view of the fact that all were T3 or T4 tumours. The degree of downstaging (DS) also correlated significantly with RFS. Table 4.7 shows all the significant univariate correlations. P53 expression in both the surgical and biopsy specimens was significant for a poorer RFS. Increasing TP expression in the surgical sample was also associated with a poorer RFS, there were no survivors at 5 years in patients whose tumours expressed TP in over 50% of tumour cells. Increasing EGFR intensity in the biopsy sample was associated with an improved RFS.

Using the Cox proportional hazards model; N stage ( $\chi^2=5.81, p=0.016$ ) degree of tumour downstaging ( $\chi^2=7.97, p=0.019$ ) EGFR biopsy intensity ( $\chi^2=10.79, p=0.049$ ) and P53 biopsy percentage ( $\chi^2=10.11, p=0.039$ ), remained significant on multivariate analysis. In particular p53 of moderate staining had a very poor RFS. In view of the number of variables in the analysis and the smaller number of patients the EGFR and p53 scoring were condensed into fewer scoring groups. This resulted in an increased statistical significance for EGFR and less significance for p53 and is likely to be a more appropriate reflection of the statistical relevance.

Figure 4.18 demonstrates the RFS survival differences for the clinicopathological variables downstaging and nodal status each of these variables and Figures 4.19 and 4.20 demonstrate the RFS differences for p53 and EGFR.

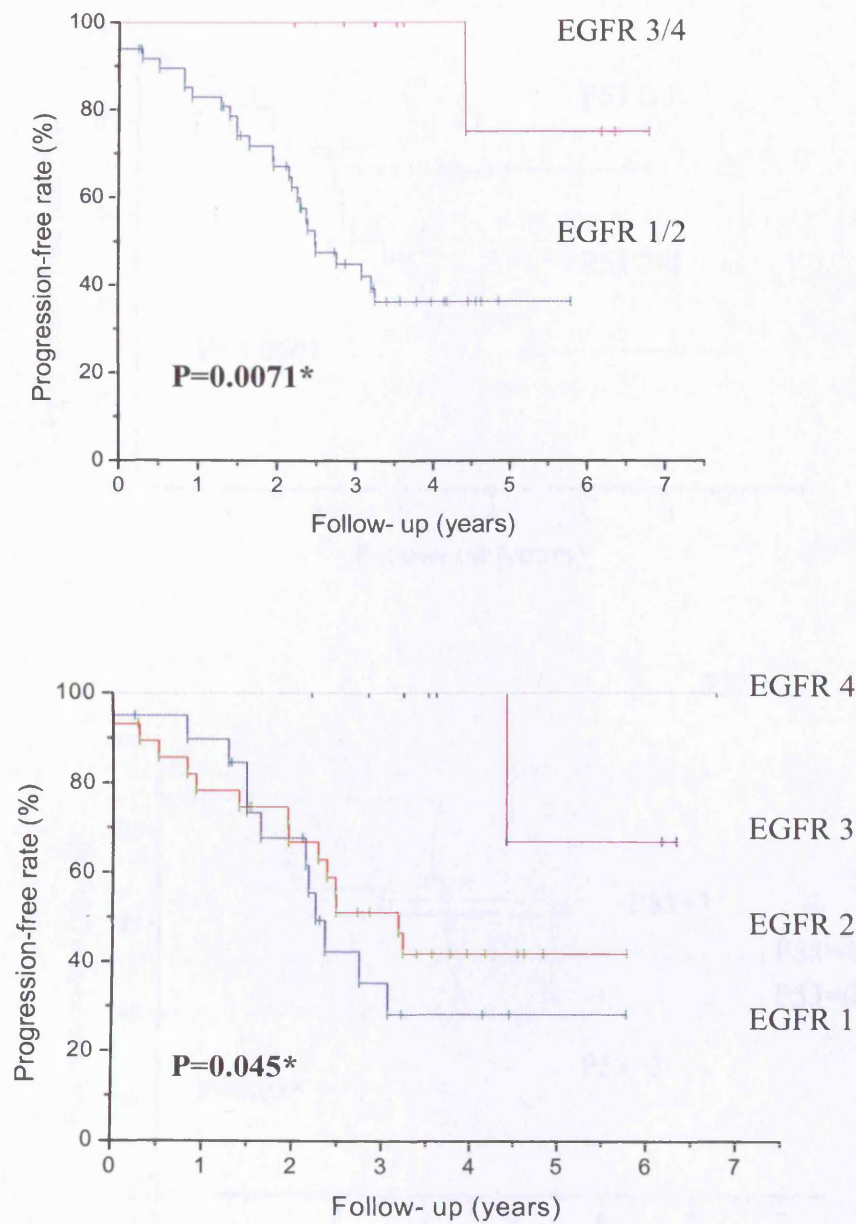
<b><u>Variable</u></b>		<b><u>Code</u></b>	<b><u>%</u></b>	<b><u>5yr RFS ± 1SE</u></b>	<b><u>95% CI</u></b>
<b>N stage:</b>	N0	0	68%	56.0% ± 10.3	35.8-76.2
	N1	1	32%	18.3% ± 9.5	-0.3-36.9
<b><u>P=0.011*</u></b>					
<b>Downstage:</b>	CPR	1	10%	80.0% ± 17.9	44.9-115.1
	DS	2	17%	88.0% ± 10.5	67.4-108.6
	None	3	73%	30.6% ± 8.7	13.5-47.7
<b><u>P=0.018*</u></b>					
<b>EGFR Int:</b> (biopsy)	None	0	35%	28.0% ± 11.5	5.5-50.5
	Weak	1	48%	41.0% ± 10.0	21.4-60.6
	Moderate	2	15%	66.6% ± 27.2	13.3-119.9
	Strong	3	2%	100%	
<b><u>P=0.045*</u></b>					
<b>P53 Int:</b> (biopsy)	None	0	34%	61.1% ± 11.5	38.6-83.6
	Weak	1	5%	None	None
	Moderate	2	13%	None	None
	Strong	3	48%	50.9% ± 12.0	27.4-74.4
<b><u>P=0.034*</u></b>					
<b>P53%:</b> (biopsy)	0-5%	0	34%	62.0% ± 11.4	39.7-84.3
	5-20%	1	10%	100%	
	20-50%	2	23%	15.5% ± 20.4	-24.5-55.5
	50-75%	3	13%	52.5% ± 20.4	12.5-92.5
	>75%	4	20%	43.2 % ± 20.8	3.2-83.2
<b><u>P=0.02*</u></b>					
<b>TP%:</b> (surgery)	0-5%	0	58%	38.1% ± 11.6	15.4-60.8
	5-20%	1	24%	65.6% ± 20.9	24.7-106.5
	20-50%	2	15%	40.0% ± 21.9	-2.9-82.9
	>50%	3	3%	None	None
<b><u>P=0.019*</u></b>					

Table 4.7 Log rank univariate analysis RFS and significant indices

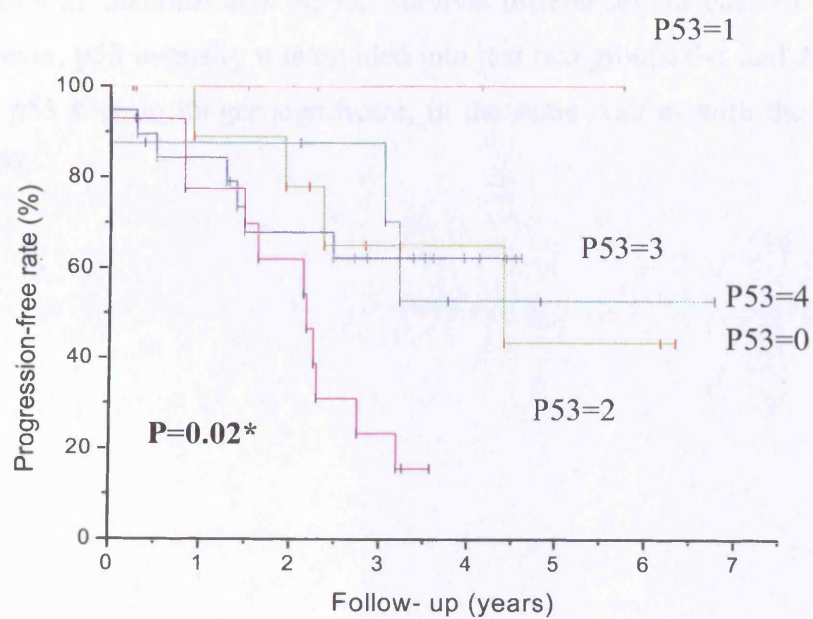
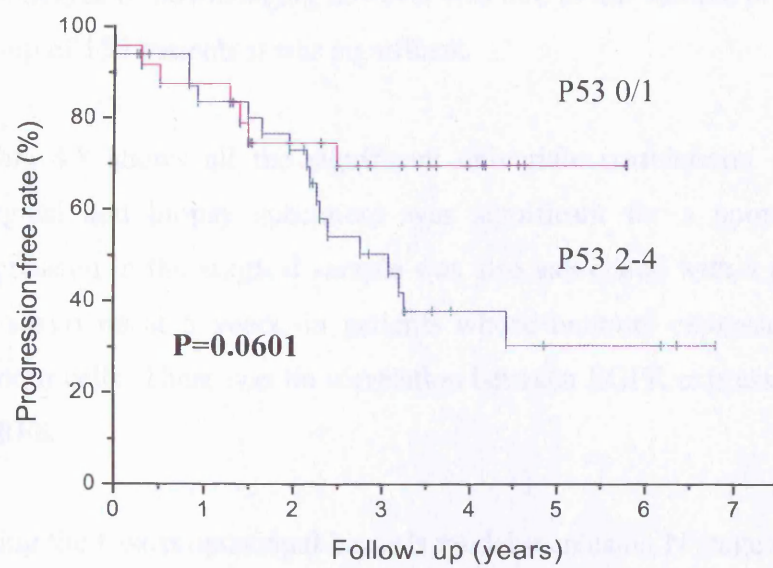


**Figure 4.18** Relapse-free survival for nodal status (upper) and degree of downstaging (lower)





**Figure 4.19** Relapse-free survival for EGFR expression, scored 1-2 (upper) and 1-4 (lower)



**Figure 4.20** Relapse-free survival for p53 expression, scored 0-1 (upper) and 2-4 (lower)

#### **4.8.8.2 Overall survival**

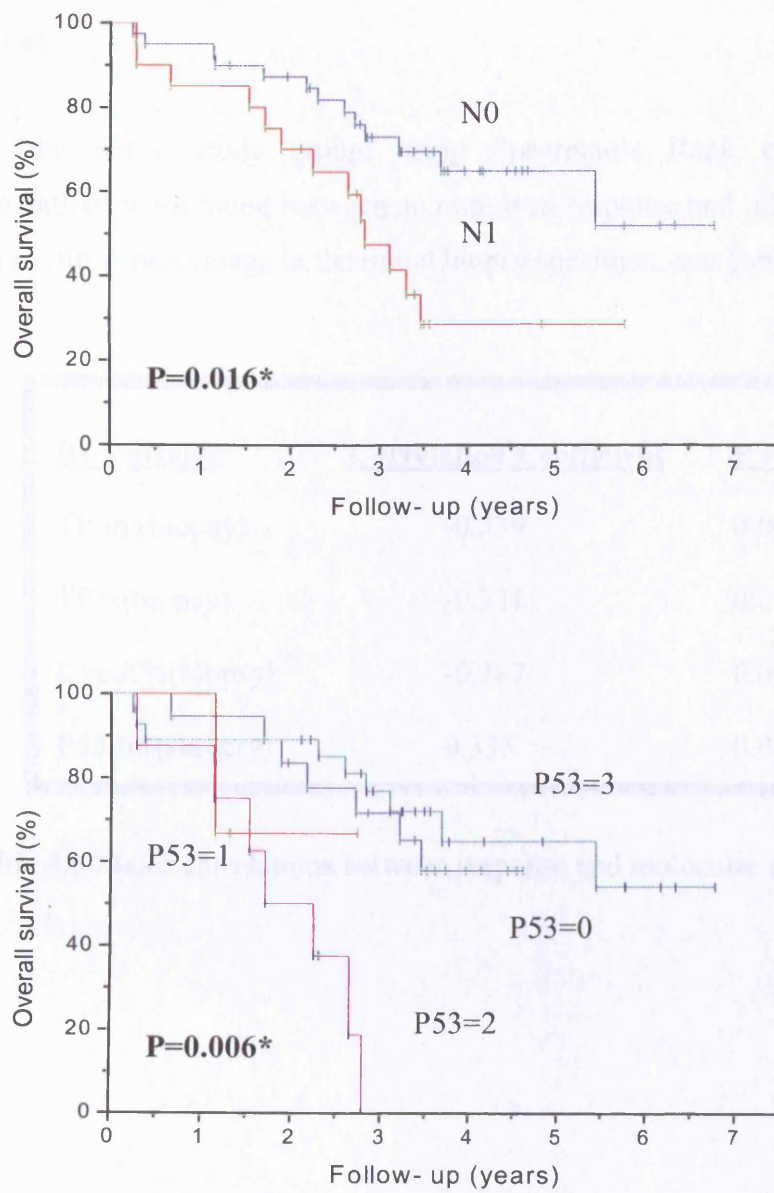
On univariate analysis increasing N stage was statistically significant for a poorer OS. The degree of downstaging however was not, in this sample of 60 patients, but for the group of 155 patients it was significant.

Table 4.8 shows all the significant univariate correlations. P53 expression in the surgical and biopsy specimens was significant for a poorer OS. Increasing TP expression in the surgical sample was also associated with a poorer OS. There were no survivors at 5 years, in patients whose tumours expressed TP in over 20% of tumour cells. There was no correlation between EGFR expression and OS, in contrast to RFS.

Using the Cox proportional hazards model increasing N stage ( $\chi^2=5.26$ ,  $p=0.022$ ) and p53 biopsy intensity ( $\chi^2=8.21$ ,  $p=0.041$ ) remained significant on multivariate analysis. Figure 4.21 demonstrates the OS survival differences for each of these variables. If However, p53 intensity was divided into just two groups 0-1 and 2-3, the association with p53 was no longer significant, in the same way as with the RFS analysis ( $p=0.905$ ).

<u>Variable</u>		<u>Code</u>	<u>%</u>	<u>5yr RFS ± 1SE</u>	<u>95% CI</u>
<b>N stage:</b>	N0	0	68%	64.6% ± 8.6	47.8-81.4
	N1	1	32%	28.4% ± 11.1	6.7-50.1
<b><u>P=0.016*</u></b>					
<b>P53 Int:</b> (biopsy)	None	0	34%	57.7% ± 12.4	33.4-82.0
	Weak	1	5%	66.6% ± 27.2	13.3-119.9
	Moderate	2	13%	None	None
	Strong	3	48%	64.6% ± 10.7	43.7-85.5
<b><u>P=0.006*</u></b>					
<b>TP%:</b> (surgery)	0-5%	0	58%	42.0% ± 12.5	17.5-66.5
	5-20%	1	24%	87.5% ± 11.7	64.6-110.4
	20-50%	2	15%	None	None
	>50%	3	3%	None	None
<b><u>P=0.006*</u></b>					
<b>P53 Int:</b> (surgery)	None	0	39%	62.6% ± 13.5	36.1-89.1
	Weak	1	0%	0	
	Moderate	2	22%	19.1% ± 16.8	-13.8-52.0
	Strong	3	39%	36.7% ± 17.6	2.2-71.2
<b><u>P=0.025*</u></b>					

**Table 4.8** Log rank univariate analysis OS and significant indices



**Figure 4.21** Overall survival for nodal status (upper) and p53 expression, (lower)

#### 4.8.8.3 Response & correlation with other parameters

Downstaging in response to chemoradiation has already been shown earlier in this chapter, to be a significant marker of both OS and RFS in locally advanced rectal cancer.

For the entire study group, using Spearman's Rank coefficient, significant associations were found between an improved response and increasing TP expression and cyclin A percentage in the initial biopsy specimen, see Table 4.9.

<u>By Variable</u>	<u>Correlation Coefficient</u>	<u>P value</u>
TP Int(biopsy)	-0.339	0.008
TP%(biopsy)	-0.331	0.010
Cyc A%(biopsy)	-0.287	0.028
P53 Int(surgery)	0.335	0.046

**Table 4.9** Major correlations between response and molecular markers

## 4.9 Discussion

Following a curative surgical resection, the prognosis of rectal cancer remains poor. 5-year survival rates fall from 85% in stage I disease to 60% in stage II and 40% in stage III [285]. First recurrence sites in stage II and III cancer occur to an almost equal extent in the pelvis, liver and lung [286]. Because of the anatomical constraints of the pelvis, achieving local control while preserving sphincter function can be challenging. Local recurrence rates can be as high as 25% and they are associated with significant morbidity [51, 287-291]. The social and psychological consequences of a permanent colostomy are obvious.

The difficulty in obtaining a negative radial resection margin is one of the main causes of local recurrence [292-295]. Multivariate analyses have suggested that tumour involvement of the radial margin may be the most critical single factor in predicting local recurrence in rectal cancer [294]. One recent technique, which has improved local control rates by reducing the incidence of a positive CRM, is total mesorectal excision. Local recurrence rates of less than 10% have been reported with this procedure, in selected patients [40].

There has been considerable oncological interest in ways of further improving the local recurrence rates and survival in rectal cancer. Adjuvant radiation with or without 5FU based chemotherapy delivered before or after surgery has been demonstrated to improve local control in patients with high risk rectal cancers [51, 287-289] and pre-operative radiotherapy has been used successfully as an adjuvant to surgery, providing good local control and potentially enhancing sphincter preservation rates [42, 51, 287-289, 291, 296]. Initial experience with pre-operative treatment was with radiation alone. Several studies compared pre-operative radiotherapy at moderate doses (25-40Gy) to surgery only [42, 297-303]. Analysis showed a significant reduction in local recurrences. Three of these studies also demonstrated a survival benefit. In a meta-analysis, the benefit in terms of local control with preoperative radiotherapy was much greater than that observed with postoperative radiotherapy [304]. The addition of chemotherapy reduces local recurrences even further [305] and toxicity rates and compliance do not seem to be significantly affected [306]. A recent randomised phase III trial has confirmed [307]

an improvement in local control with preoperative chemoradiation compared to postoperative. Almost 800 patients were randomised to either preoperative or postoperative chemoradiation in T3/4 rectal tumours. There was a significant improvement in local control ( $P=0.02$ ) and in improved sphincter preservation in low rectal tumours ( $P=0.004$ ). The results of the EORTC 22921 are also awaited, which has randomised patients between preoperative radiotherapy alone versus preoperative chemoradiation..

Preoperative treatment potentially downstages locally advanced tumours such that a negative resection margin can be achieved with surgery. The preoperative approach also has several other potential benefits, enabling an enhanced effect on micrometastatic disease and an enhanced compliance, since the treatment is administered to previously untreated patients. Sauer et al [307] showed a trend for reduced acute toxicity and significantly less chronic toxicity at the anastomotic site for preoperative treatment.

The purpose of this study was to analyse outcome data, in conjunction with tumour molecular marker expression in a group of patients with locally advanced carcinoma of the rectum, treated with pre-operative 5FU based chemoradiation prior to definitive curative surgery. All these patients underwent chemoradiation for the purpose of downstaging, as the referring surgeons felt there was a high risk of achieving an R1/2 resection margin.

The first part of the analysis was to confirm the previously reported poor survival in this group of patients [285] and determine the incidence of local recurrence and metastatic disease. Only 31% of patients in this current series were disease-free at 5 years. The median time to the development of a local recurrence was 22 months and 19 months for distant metastases. Therefore, at least half the recurrences occurred in the first two years following treatment. The overall median survival for the group was 37 months and only 34% were alive at 5 years of follow-up. This confirms that this group of patients does have a poor survival with high incidences of both local recurrence and metastatic disease.



The importance of a positive circumferential (CRM) margin (R1/2) in determining the incidence of local recurrence has been noted earlier. In this analysis data on the surgical resection margins had been collected on all the patients who underwent surgery. It was therefore possible to determine outcome according to the resection margin as defined by UICC criteria. From the original group of 155 patients, 122 patients at the time of this analysis had undergone surgery with curative intent. A negative CRM was achieved in 80% of surgical cases giving an overall negative CRM for the whole group of 63%. The importance of a positive CRM became evident in the recurrence and survival analysis. Only 10% of patients, who had a negative CRM, developed a local recurrence, in comparison to 62% in the positive CRM group. Similarly, 29% of patients in the negative CRM group developed distant metastases compared to 75% in the positive CRM group. This resulted in a significant difference in terms of both relapse-free survival ( $P < 0.001$ ) and overall survival ( $P = 0.0001$ ) between the two groups. At 3 years 52% of patients with a negative CRM were relapse-free compared to 9% in the positive CRM group and overall survival at 3 years was 64% vs. 25%. It is therefore important in this group of patients to achieve where possible a negative circumferential margin. A recent phase II trial, Socrates [308], has reported negative CRM's of greater than 80% using a combination of oxaliplatin and 5FU and similar trials looking at irinotecan and 5FU are underway.

The incidence of a complete pathological response was 12% with pathological downstaging to a pT0/1/2 tumour in 28% of cases, this is in line with other reported series [307, 309]. Response to chemoradiation was important in terms of survival and this has also been found previously [135, 173]. For this series, the patients were divided into those whose tumours had been pathologically downstaged and those whose tumours had not. There was a significant improvement in terms of RFS ( $P = 0.0032$ ) and OS ( $P = 0.020$ ) in those who were downstaged. This may in part be a reflection of a good response to chemoradiation acting as a surrogate marker for achieving a negative CRM. If response to chemoradiation can predict outcome as in this series, can a complete pathological response predict for an even greater improvement in survival outcomes. This has been postulated but there have been no trials which have established this. Rich et al [53] reported a non-significant improvement in patients with a CPR but did not report local recurrence data. In this

series of patients this was not the case. Patients with a CPR did not do better than the group of patients who had been pathologically downstaged.

The main prognostic factors in rectal cancer remain the extent of the primary tumour (T stage), regional lymph node status (N stage) [310], the histological grade, the presence of vascular invasion and whether there is a sufficient circumferential resection margin. These factors however, are not easy to predict preoperatively. Thus there have been considerable efforts to define accurate histological, pathological and molecular information preoperatively, which have a bearing on proliferation, apoptosis and other molecular targets and can hence predict future outcome. Presently little is known about pre-treatment characteristics that may predict response to chemoradiation in patients with rectal cancer. For this reason the second part of this study analysed a panel of biological molecular markers, which in previous reported series of colorectal carcinomas have been implicated as predictive markers of treatment response, outcome or both. From the initial series of 155 patients, pre-chemoradiation biopsy specimens were received on 60 patients and from these; surgical operative specimens were also received on 33 patients. Six markers were chosen to reflect previous published series and to give a biological overview of the different processes involved in tumour growth, cell cycle regulation and angiogenesis.

#### **4.9.1 Correlation between molecular markers and clinicopathological variables**

Analogous to the anal carcinoma series, the rectal samples expressed a high rate of the proliferation marker cyclin A and over half (52%) were bcl-2 positive; this is in line with other reported series [110, 117, 135, 145]. Over two thirds (67%) were p53 positive, which is slightly higher than some quoted series of 40-50% [107, 113, 118, 121, 122, 145, 151]. However, only 60% expressed TP and, unlike in the anal carcinomas, the expression was predominantly in the stroma. Over 90% of tumours expressed TS with considerable variation in the percentage of tumour cells staining positively. This degree of expression is higher than some reported series of 69% [171] and 65% [173]. In this present study 65% of tumour samples were EGFR positive but the staining was cytoplasmic with very little membrane expression.

T stage correlated with increasing cyclin A percentage and TS, both markers of proliferation. Cyclin A also correlated with increasing tumour grade indicating that

the higher grade and T stage tumours are proliferating more rapidly. Proliferative activity has been shown previously to be correlated with tumour stage in colorectal carcinoma [279].

P53 has also been shown to correlate with T stage [113] however in this analysis p53 did not correlate significantly with any of the clinicopathological variables. This is not surprising as only locally advanced tumours were in the analysis. This lack of association has however, been found in other rectal cancer series [151].

Bcl-2 in previous reported studies has correlated with stage and lymph node status [117, 311], this correlation was found in this analysis. A significant association was also found between TS, cyclin A, bcl-2 and age. This association between TS and age has been demonstrated previously [312]. The explanation for this is unclear.

In this series, a negative correlation between increasing N stage and increasing TP was found. This negative correlation has been found in other series, including breast carcinomas, [210] postulating that TP mediates its angiogenic activity early in breast carcinoma progression by remodelling the existing vasculature within the affected breast lobules, as high TP expression was also found in low-grade invasive breast carcinomas. This hypothesis may also explain the association seen in this study. However this is contradicted in previous studies [313] in which TP expression has correlated with both the size of the tumour and the incidence of lymph node metastasis.

#### **4.9.2 Correlations between molecular markers**

When the correlations between the markers were studied p53 staining correlated significantly with TS staining. This association has been noted in a series of 25 patients with stage II colon cancer, in which nuclear expression of p53 was observed to be associated with high levels of TS and 91% of recurrences occurred in patients whose tumours had mutated p53 and high levels of TS [314]. An interaction between TS and p53 occurs at the translational level as TS can bind to the mRNAs of both p53 and c-myc [315, 316]. This finding is important because it suggests that mutated p53 may increase TS levels and cause resistance to chemotherapy. However, TS expression in this series of rectal carcinomas was not prognostic for either survival

outcome in multivariate analyses. TS also strongly correlated with TP, unlike in the anal carcinoma series. This suggests in the rectal carcinomas the 5FU markers may play a more significant role in influencing response.

Positive correlations between TP and bcl-2 expression have been shown previously and both were inversely correlated with increasing tumour grade [210]. This association between grade and bcl-2 was found in the present series and TP was also strongly correlated with p53, which has been addressed previously [203] suggesting that TP expression might be modulated by p53. Shomori's study [203] found that the frequency of TP expression in colorectal carcinomas was significantly higher in those with p53 expression, than in those without.

EGFR significantly correlated with p53, TS and TP. This association has not been demonstrated previously. Binding to the extra-cellular domain of EGFR causes the activation (phosphorylation) of a number of downstream effectors involved in the *ras/raf-1/mitogen-activated protein kinase*, and the *phatidylinositol-3-kinase*, and the *phospholipase C $\gamma$*  pathways [317]. Activation of these cascades ultimately results in the transcription of other genes responsible for cell growth co-ordination, regulating cell division, proliferation and differentiation. This may well explain the associations with TP, TS and p53 seen in this study.

Another factor analysed in this study was the difference in expression of each of the molecular markers pre and post chemoradiation. Previous reported series of Ki-67 in rectal carcinomas have demonstrated that expression in the surgical specimen was lower than in the biopsy specimen [130, 131, 133]. This was also true of cyclin A in this series with 22% of pre-chemoradiation biopsy specimens being negative for cyclin A and 40% negativity in the surgical specimens. It is not unexpected that a course of CRT will alter cell kinetics. The therapy should preferentially affect rapidly dividing cells, leaving behind a population biased toward slow proliferation [318].

P53 expression did not alter significantly between the biopsy and the surgical specimens. Previous studies have demonstrated that [106] p53 is upregulated in normal human cells after short course pre-operative RT but not in rectal tumour cells.

This indicates that the p53 in rectal tumours does not respond to radiation, suggesting a high frequency of p53 abnormalities.

In this study EGFR was upregulated in response to chemoradiation. Lammering et al have demonstrated that the exposure of tumour cells to ionising radiation in the therapeutic dose range (1-5Gy) results in the immediate activation of EGFR [319] and that repeated radiation exposures of 2Gy lead to increased EGFR expression. Bcl-2 expression also increased following chemoradiation. This may be expected as most chemotherapeutic agents and radiation, induce cell death through the triggering of the apoptotic pathway [320].

Several associations were identified between the surgical markers, which were not seen in the biopsy specimen markers. However, the sample group was much smaller and their correlations therefore may not be as significant. EGFR correlated with increasing N stage, suggesting a more advanced stage of presentation with increasing expression of EGFR. EGFR is often expressed at high levels in human cancer and has been associated with more aggressive tumours. Cyclin A correlated with TS both of which are proliferation markers, and cyclin A also correlated with bcl-2 which may be explained by their upregulation in response to chemoradiation.

#### **4.9.3 Survival outcome**

The pathological stage after complete surgical resection is probably the most important prognostic factor in rectal cancer [321]. It also determines the appropriateness of adjuvant treatment. In this study T stage was not prognostic of outcome and this is likely to be because all the tumours were locally advanced. N stage however was of prognostic significance, predicting for both a poorer RFS and OS on multivariate analyses. The database did not extend to histopathological detail with reference to the presence of perineural invasion or extra-mural extension, so the prognostic effect of these histopathological variables was not assessed.

The evaluation of grade is largely subjective and no one grading system has been widely accepted and uniformly employed. In this series grade was reported as well, moderate or poorly differentiated on the basis of the degree of glandular formation, presence of mitoses etc. It has been noted in the literature that the pathologic

diagnosis of poorly differentiated or undifferentiated tumours is relatively consistent, but discrimination between well and moderately differentiated carcinomas is associated with a significant degree of interobserver variability [322]. Despite the absence of standardised assessment, it has repeatedly been shown on multivariate analyses that grade is a stage dependent prognostic factor [323-328]. However, in this series the grade of tumour did not correlate with any of the outcome indices; this may be due to the fact that all the tumours were locally advanced in stage.

No association between the proliferation markers was observed with regard to survival. Similarly bcl-2 expression did not correlate with survival, which is in direct contrast to a study [117] of 160 rectal carcinomas in which bcl-2 expression was associated with a longer disease free interval and was an independent predictive factor of recurrence.

P53 staining of moderate intensity however, did predict for a poorer RFS ( $P=0.039$ ) and OS ( $p=0.041$ ). This result however may be due to sample size and the number of staining variables rather than a true association. The prognostic significance of p53 has been identified in other rectal carcinoma studies receiving different treatment modalities, including surgery alone, pre-operative radiotherapy and CRT, [112-114, 117, 329]. In a study [111] of pre-operative RT, the presence of a p53 mutation was associated with a significantly shorter 5 year survival and in a series of 166 resected Dukes' B2 [330] rectal carcinomas, overexpression of p53 was significant on multivariate analysis for an increased risk of recurrence. The results however, remain conflicting with several studies reporting negative results [119-122].

TS expression in this study did not predict for clinical outcome. The studies to date also show conflicting data regarding the prognostic significance of TS. Its importance is predominantly governed by whether treatment has included 5FU, because TS overexpression has been shown to correlate with high rates of recurrence and decreased survival in those treated with 5FU [170, 171]. In one previously reported study [171] 49% of patients with low TS levels were free of disease at 5 years compared to 27% in those with high TS levels. A high TS protein expression has also been shown to predict for a poorer disease-free survival and overall survival independently of Dukes stage and 5FU treatment [169, 314]. However, many series in

colorectal carcinoma do not report an association between TS and clinical outcome or subsequent response to 5FU chemotherapy [312, 331].

EGFR is being increasingly investigated as a potential marker of prognosis in several carcinoma sites. There is evidence to support that the overexpression of EGFR is an independent prognostic factor in squamous cell carcinomas of the head and neck [332-335]. In this current series of 60 rectal carcinoma patients the presence of EGFR expression correlated significantly with increasing N stage, suggesting a role in the progression of disease. There are few previous reported studies of EGFR in rectal cancer. A recent study of 45 patients who received either preoperative radiotherapy alone or preoperative 5FU-based chemoradiation, showed that increased EGFR expression correlated with a poor prognosis. However, this series recruited a mixed group of patients receiving two different pre-operative treatments.

The interesting finding in this current series is that although EGFR correlated with increasing N stage, DFS improved with increased EGFR expression. This may be due to an enhanced radiosensitisation effect. Lammering et al [319] have shown that EGFR is upregulated in response to radiation and this radiation-induced EGFR activation resulted in a pronounced dose-dependent, proliferative response that could be quantified in vitro after both single and repeated radiation exposures. They hypothesise that radiation-induced EGFR activation contributes, at least in part, to the mechanism of accelerated repopulation. This cellular proliferation response during repeated radiation exposures, as used in clinical radiotherapy, can lead to increased renewal of tumour clonogens. This has led to the concept that disruption of EGFR function should prevent the repopulation response and mediate tumour cell radiosensitisation. In a recent study in head and neck cancer the addition of cetuximab, an EGFR inhibitor, with radiation led to an improved clinical outcome [336]. Indeed, radiosensitivity was shown to be enhanced in a dominant-negative EGFR mutant, EGFR-CD533, after both single and repeated radiation exposures [319].

#### **4.9.4 Downstaging**

Increased cyclin A expression correlated strongly with improved downstaging of the primary tumour. This association between increased expression of proliferation

markers and response has been noted previously in colorectal carcinomas. In rectal cancer fast proliferating tumours have been shown to demonstrate a high immediate response to high dose radiation therapy [130] and an improved response to chemoradiation [131, 132].

P53 expression correlated strongly with a poorer response to chemoradiation and this has been noted previously in other rectal cancer studies [108, 109]. It has been suggested that this is due to increasing radio- and chemoresistance by p53 gene mutations [283, 337]. One study found that P53 expression correlated inversely with response to chemoradiation and a CPR, and directly with an increased likelihood of residual cancer in the lymph nodes of the surgical specimens [107]. However no significant correlation has been noted in other rectal carcinoma series [110, 338]. The postulated resistance to the treatment modalities of chemotherapy and radiotherapy, which has been discussed in the previous chapter may well explain the finding by Goh [115], who demonstrated a lower likelihood of benefiting from adjuvant therapy in colorectal carcinoma when a mutation in the p53 gene was present.

In this current study TS did not appear to predict for tumour downstaging. This contradicts previous reports in which [110, 173], TS expression did predict for a poorer response to 5FU CRT, however this is likely to be due to the known interactions of TS with 5FU; as the lack of TS expression was only associated with tumour downstaging after combined CRT and not with just radiation alone. Although no association between TS and response to chemoradiation in this study was found, increasing TP expression did predict for an improved response. A plausible explanation could be attributed to the angiogenic properties of the protein resulting in increased chemotherapy delivery to the tumour. Alternatively, it may also be a result of increasing sensitivity to 5FU, which has been demonstrated in a study [263] of breast carcinomas, in which patients with TP-positive tumours had a significant survival benefit when treated with 5FU combination chemotherapy. Therefore several studies, including the present one suggest that tumour levels of TP may give an indication of patient response to treatment.

This study confirms that this group of locally advanced rectal carcinoma patients have a poor outcome. The importance of achieving a negative circumferential margin is



demonstrated. Higher rates of a negative CRM can be achieved by the use of pre-operative long course 5FU-based chemoradiation. If pathological downstaging is achieved there is a significant improvement in both DFS and OS. However, further efforts are needed to improve patient outcome. This has led to the search for potential prognostic molecular markers, which could help identify patients who are likely to have a poor response to treatment or outcome. Although this is a small series several markers do appear attractive in this respect but larger randomised controlled series are required to clearly define their role in this disease site.

## **Chapter 5:**

### **The Effect of Radiation on Thymidylate Synthase and Thymidine Phosphorylase**

## **Chapter 5**

# **Assessment of the effect of radiation on thymidine phosphorylase and thymidylate synthase enzyme activity in HT29 and SW48 colon cancer cell lines**

### **5.1 Aims**

- Investigate HT29 and SW48 colon cancer cell lines in vitro and study the effect of radiation on the enzyme levels of thymidine phosphorylase and thymidylate synthase
- Establish a dose-time relationship for thymidine phosphorylase
- Establish a dose-time relationship for thymidylate synthase
- Investigate the effect of a clinical fractionated course at 2Gy per day

### **5.2 Introduction**

HT29 and SW48 are well established human colon cancer cell lines. HT29 is relatively radioresistant and has mutated p53 whilst SW48 is relatively radiosensitive and is p53 wild-type. The cell culture conditions and preparation are described in Chapter 2 as are the enzyme activity assays and protein expression of thymidine phosphorylase and thymidylate synthase. The details of the fractionation, radiation and timing of the experiments are in appendix I.

### **5.3 Single dose experiments enzyme activities**

Both cell lines were exposed to single doses of 2Gy and 10Gy. Enzyme activities and protein expression were then measured at 0, 2, 7, 24, and 48 hours after irradiation. These measurements were performed in triplicate and at each dose and time point a sham irradiation (RT) was given to a control sample.

### **5.3.1 Thymidylate synthase activity**

#### **5.3.1.1 HT29**

Figure 5.1 shows the TS enzyme activities for the three HT29 experiments in response to single doses of radiation.

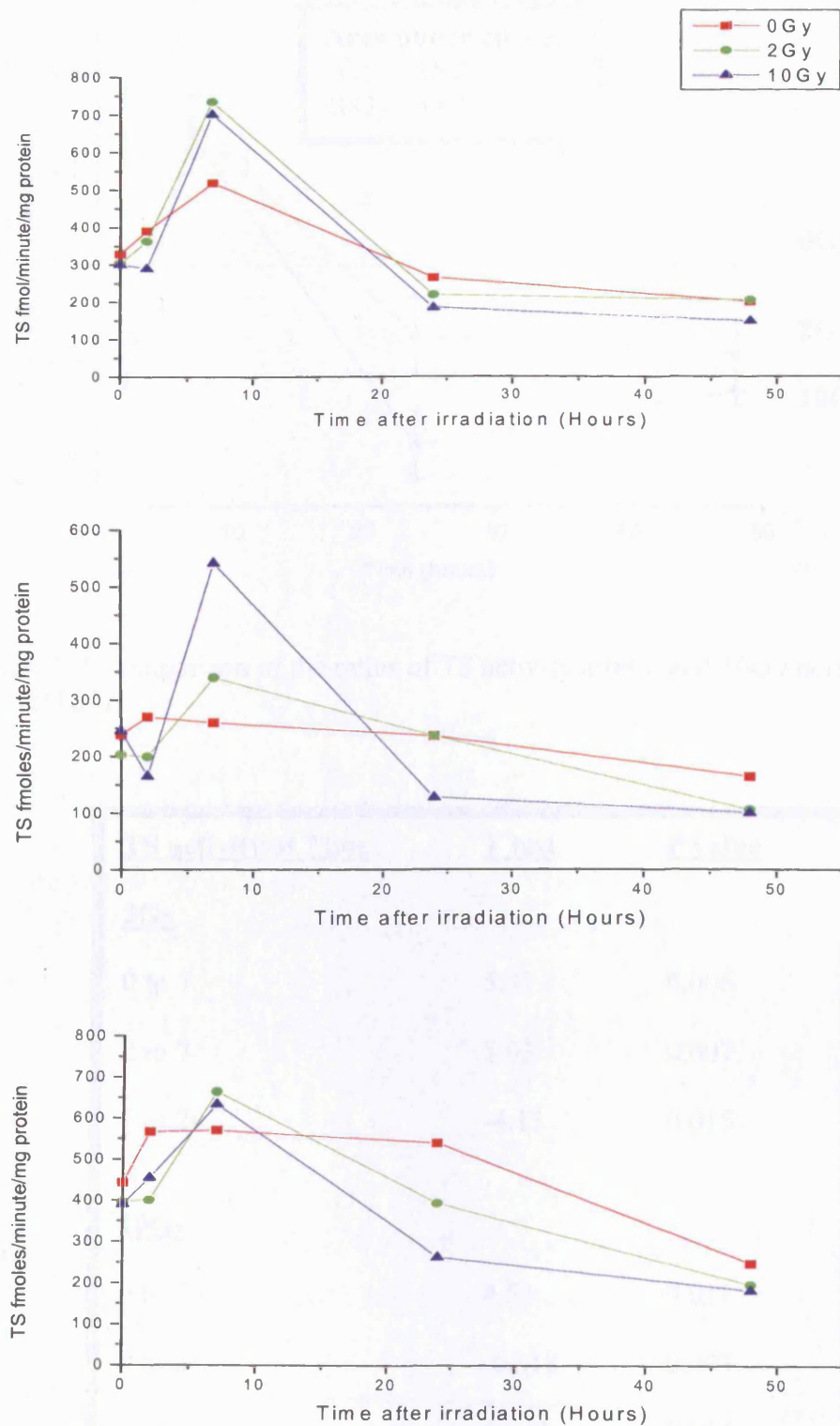
TS activity in the HT29 cells rose at 7 hours, on average to 1.5 times the control level. This change did not appear to be dose dependent with similar differences occurring at both 2 and 10Gy. TS activity then fell at 24 to 48 hours, with a more prominent drop occurring at 10Gy, to 50% of the control activity. It is interesting to note that TS levels fell in the unirradiated controls over time and as will be seen later in the flow cytometry results, this is likely to be due to an increase in cell confluence.

Figure 5.2 shows the ratio to 0Gy, of each of the time points combined from each experiment, for the 2 and 10Gy. This shows the changes in enzyme activities more clearly. It also shows that there was an initial drop in activity at 2 hours after RT.

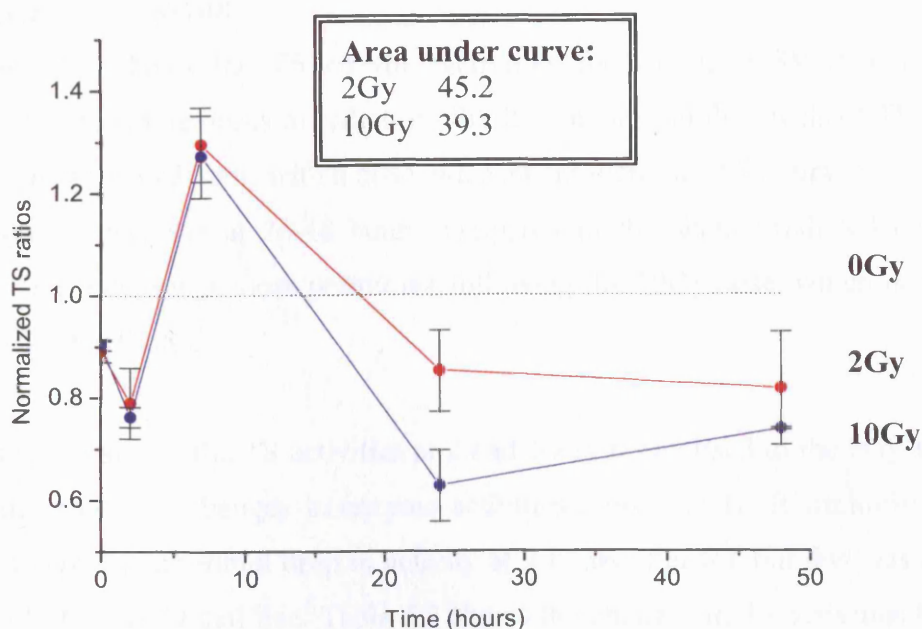
The TS activities were then analysed at each time point for each radiation dose. Table 5.1 shows the significant changes in TS activity between different time points, as measured by Anova's T test.

This shows that following a radiation dose of 2Gy and 10Gy there is a significant increase in TS levels at 7 hours and a significant decrease from 7 to 24 hours. At 10Gy there was also a significant drop overall from 0 to 48 hours.

A comparison of TS activities was also made between the time points for the different doses of RT, using the one way anova T test. There were no significant differences between the TS activities for the two different radiation doses at each time point. This is consistent in the HT29 cell line with the TS activity changes not being dose dependent.



**Figure 5.1** TS activity over time following single dose irradiation in the HT29 cell line



**Figure 5.2** Comparison of the ratios of TS activity after 2 and 10Gy normalised to 0Gy (HT29)

<u>TS activity at Time</u>	<u>T test</u>	<u>P value</u>
<b><u>2Gy</u></b>		
0 to 7	5.37	0.006
2 to 7	5.03	0.007
7 to 24	-4.13	0.015
<b><u>10Gy</u></b>		
0 to 7	4.53	0.011
0 to 2	-6.318	0.003
2 to 7	6.149	0.004
7 to 24	-6.06	0.004
0 to 48	-15.15	<0.001

**Table 5.1** Significant changes in TS activity levels in the HT29 cell line

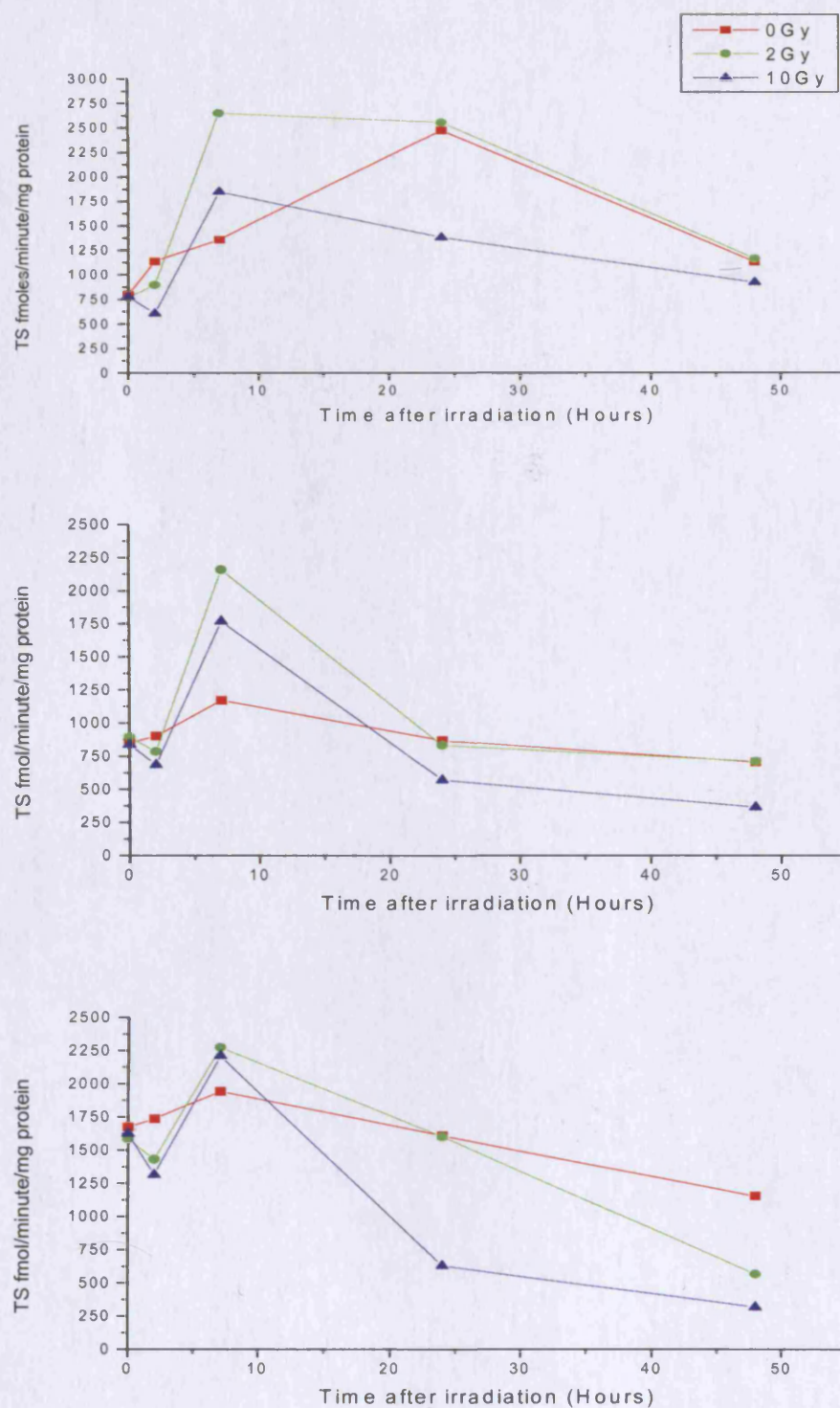
### 5.3.1.2 SW48

Figure 5.3 shows the TS enzyme activities for the three SW48 experiments in response to single doses of radiation. Similar changes in the levels of TS activity are seen in the SW48 cells, with a dose independent increase at 7 hours following RT and a gradual decrease at 24-48 hours compared to the sham irradiated controls. The decrease however is more prominent following the 10Gy dose, which is also seen in the HT29 cell line.

Figure 5.4 shows the TS activities at 2 and 10Gy normalised to the 0Gy values. This again shows the changes in enzyme activities more clearly. It similarly shows that there was also an initial drop in activity at 2 hours after RT but this was not as clear as with the HT29 cell line. Table 5.2 shows the changes in TS activities between the time points for each radiation dose, which were significant.

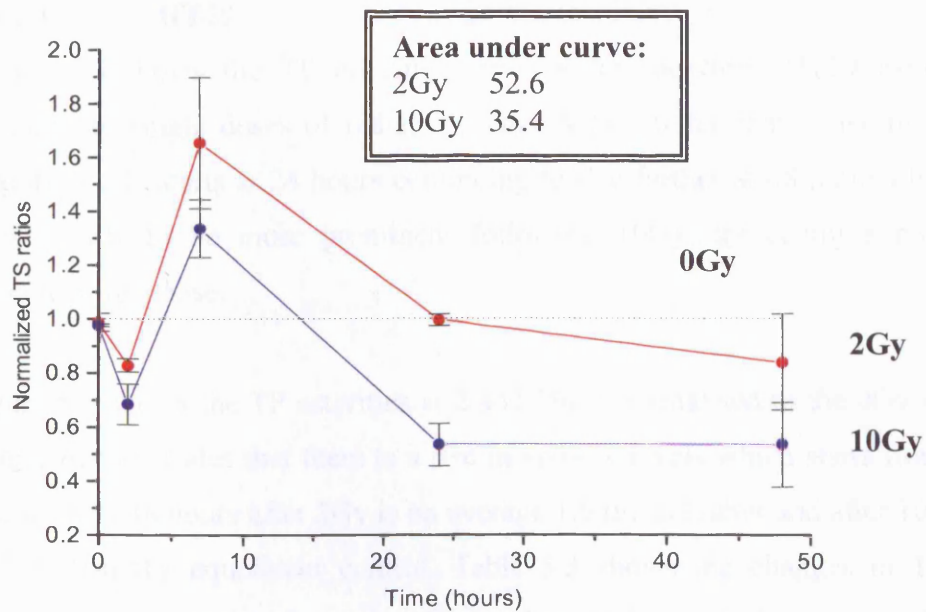
The comparison of TS activities at each time point, between the two RT doses, revealed that in SW48 there was a significant difference between the activities at 24 hours following RT (T test -6.601,  $p=0.0012$ ). The 10Gy TS activity levels at this time point were significantly lower than in the 2Gy. There was also a borderline significant difference at 2 hours with a more prominent drop in the 10Gy samples (T test -2.324,  $p=0.068$ ).

These results confirm that there is a drop in TS activity levels at 2 hours after RT, in both cell lines; however this is only statistically significant after 10Gy. This change would therefore appear to be dose dependent. There is a significant rise in TS activity levels in both cell lines at both doses of RT at 7 hours, which does not appear to be dose dependent. The final significant change is a drop in TS activity levels over 24-48 hours after RT. This again is more significant at the 10Gy dose. A difference in activity between the two different RT doses was only detectable in the SW48 cell line. It is also interesting to note that SW48 cells appear to have higher levels of TS than HT29 cells. There is no significant difference in TS activity response to RT between the two cell lines as demonstrated by the similar values for area under the curve.



**Figure 5.3** TS activity over time following single dose irradiation in the SW48 cell line





**Figure 5.4** Comparison of the ratios of TS activity after 2 and 10Gy normalised to 0Gy (SW48)

<u>TS activity at Time</u>	<u>T test</u>	<u>P value</u>
<u><b>2Gy</b></u>		
0 to 7	2.18	0.053
2 to 7	3.37	0.028
7 to 24	-2.70	0.054
<u><b>10Gy</b></u>		
0 to 7	3.32	0.030
0 to 2	-3.92	0.017
2 to 7	4.97	0.008
7 to 24	-6.01	0.004
0 to 48	-2.83	0.047

**Table 5.2** Significant changes in TS activity levels in the SW48 cell line

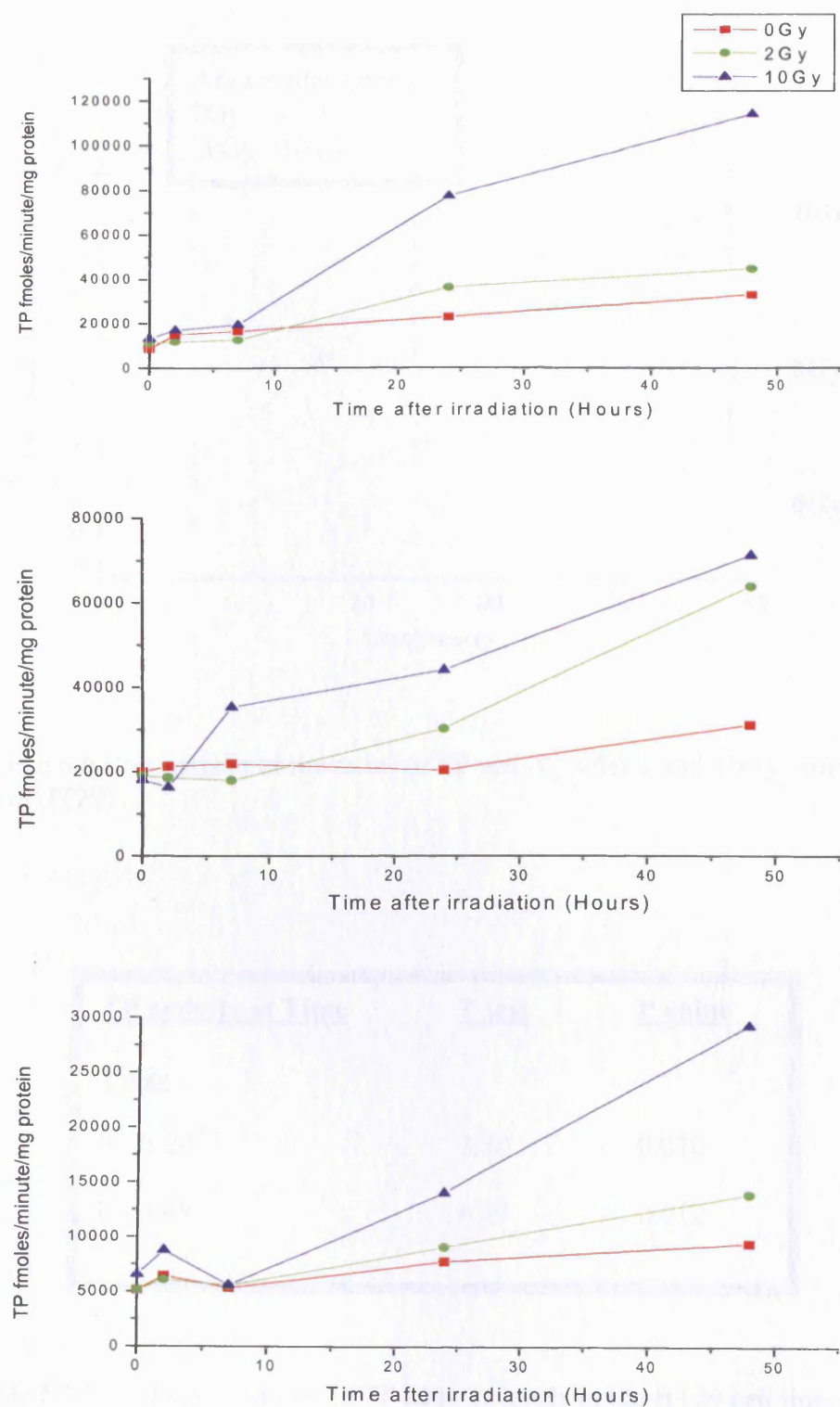
## **5.3.2 Thymidine phosphorylase**

### **5.3.2.1 HT29**

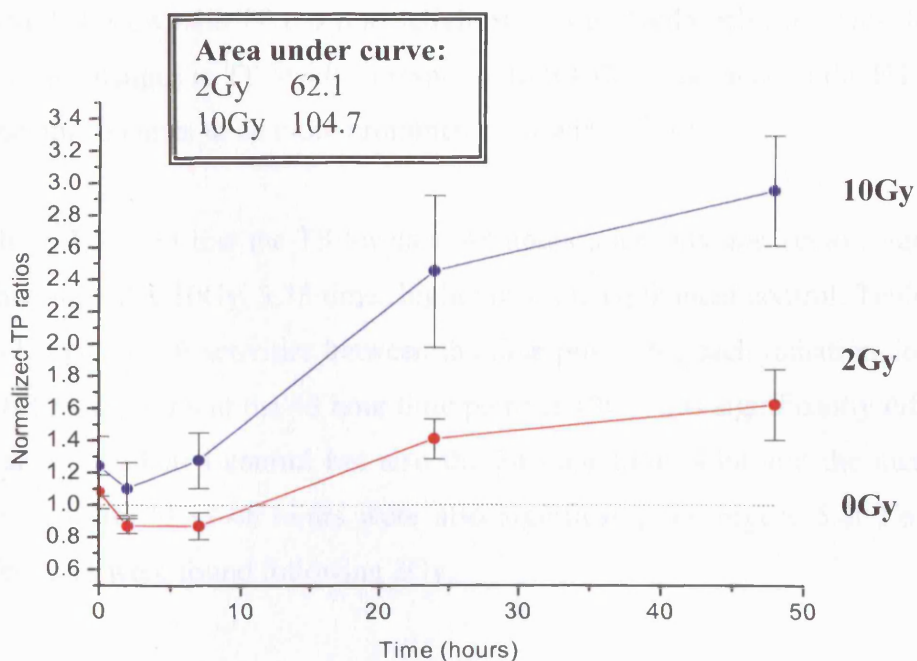
Figure 5.5 shows the TP enzyme activities for the three HT29 experiments in response to single doses of radiation. This demonstrates that a rise in TP enzyme activity levels starts at 24 hours continuing to rise further at 48 hours after RT. This does appear to be more prominent following 10Gy, indicating a possible dose dependent response.

Figure 5.6 shows the TP activities at 2 and 10Gy normalised to the 0Gy values. This clearly demonstrates that there is a rise in activity levels which starts from 24 hours. The level at 48 hours after 2Gy is on average 1.5 times higher and after 10Gy, 3 times higher than the equivalent control. Table 5.3 shows the changes in TP activities between the time points for each radiation dose. Only the 48 hour time points at 10Gy were significantly different from the sham irradiated controls. The 24 hour time point at 10Gy was of borderline significance. No significant differences were seen following the 2Gy dose.

The comparison of TP activities between the time points for the different doses of RT showed that the only significant difference in TP levels between the two doses of RT occurred at the 48 hour time point (T test 3.262,  $p=0.031$ ), with a significantly higher rise in TP activity following 10Gy. It would therefore appear that the rise in TP activity following RT may occur in a dose dependent manner in the HT29 cell line.



**Figure 5.5** TP activity over time following single dose irradiation in the HT29 cell line



**Figure 5.6** Comparison of the ratios of TP activity after 2 and 10Gy normalised to 0Gy (HT29)

<u>TP activity at Time</u>	<u>T test</u>	<u>P value</u>
<u>10Gy</u>		
0 <sup>0</sup> to 24 <sup>0</sup>	2.38	0.070
0 <sup>0</sup> to 48 <sup>0</sup>	4.39	0.012

**Table 5.3** Significant changes in TP activity levels in the HT29 cell line

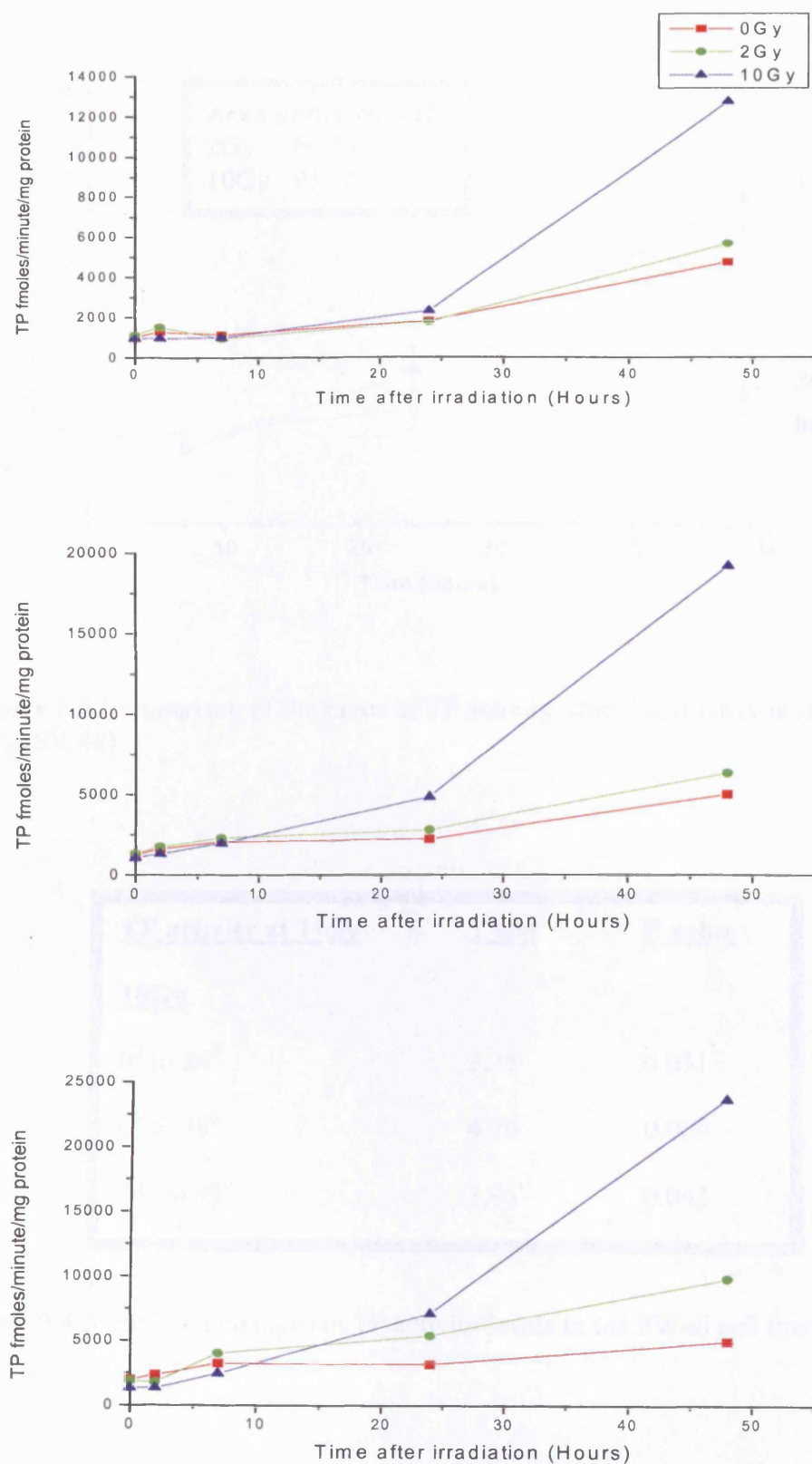
### 5.3.2.2 SW48

Figure 5.7 shows the TP enzyme activities for the SW48 cell line. This demonstrates the same changes in TP levels in response to RT that were seen in the HT29 cell line. Again this appears to be more prominent following 10Gy.

In the SW48 cell line the TS levels at 48 hours after 2Gy was on average 1.5 times higher and after 10Gy, 3.75 times higher than the equivalent control. Table 5.4 shows the changes in TP activities between the time points for each radiation dose. As with the HT29 experiment the 48 hour time point at 10Gy was significantly different from the sham irradiated control but also the 24 hour time point and the increase in TP activity from 24 to 48 hours were also significant, see Figure 5.8. No significant differences were found following 2Gy.

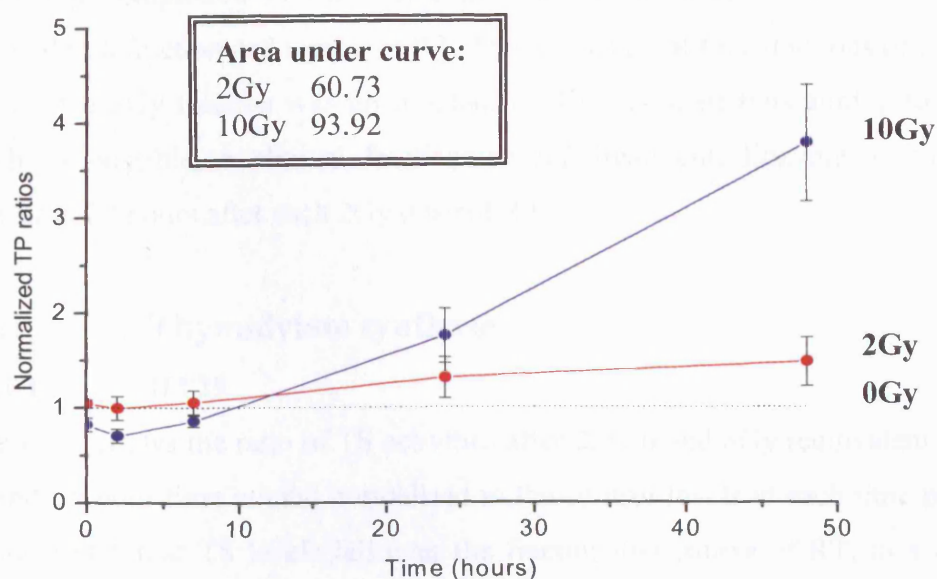
The comparison of TP activities between the time points for the different doses of RT showed that the only significant difference in TP levels between the two doses of RT occurred at the 48 hour time point (T test 3.37,  $p=0.028$ ). This finding is the same in the HT29 cell line demonstrating that the rise in TP activity following RT may occur in a dose dependent manner in both cell lines.

These results confirm that there is a significant rise in TP activity after RT, in both cell lines. This becomes most apparent at 10Gy and is statistically significant at the 48 hour time point. This change would therefore appear to be dose dependent. There also appears to be a higher TP enzyme activity in the HT29 cell line compared to the SW48 cell line but no significant difference in TP activity response to RT between the two cell lines was demonstrated, which can be seen from the similar values for area under the curve.



**Figure 5.7** TP activity over time following single dose irradiation in the SW48 cell line





**Figure 5.8** Comparison of the ratios of TP activity after 2 and 10Gy normalised to 0Gy (SW48)

<u>TP activity at Time</u>	<u>T test</u>	<u>P value</u>
<b><u>10Gy</u></b>		
0 <sup>0</sup> to 24 <sup>0</sup>	3.25	0.031
0 <sup>0</sup> to 48 <sup>0</sup>	4.79	0.009
24 <sup>0</sup> to 48 <sup>0</sup>	2.96	0.042

**Table 5.4** Significant changes in TP activity levels in the SW48 cell line

## **5.4 Fractionated experiments enzyme activities**

Following completion of the single dose experiments both cell lines were then exposed to a fractionated course of RT. This consisted of four fractions of 2Gy over 4 days. Each 2Gy fraction was given exactly 24 hours apart thus aiming to mirror as much as possible, a clinical fractionated RT treatment. Enzyme activities were measured 24 hours after each 2Gy dose of RT.

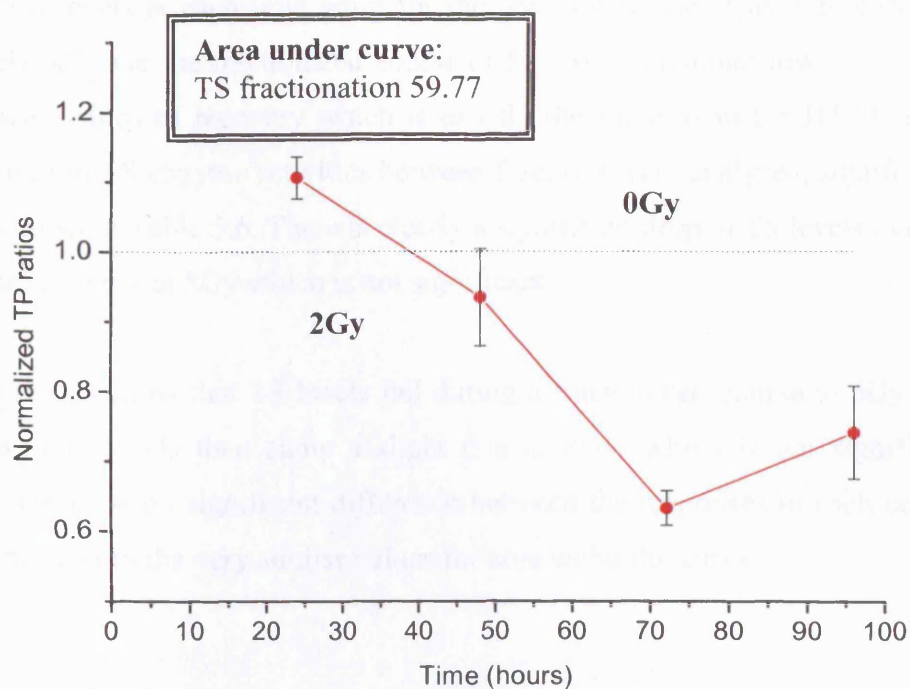
### **5.4.1 Thymidylate synthase**

#### **5.4.1.1 HT29**

Figure 5.9 shows the ratio of TS activities after 2, 4, 6 and 8Gy (equivalent to 24, 48, 72 and 96 hour time points) normalised to the control levels at each time point. This demonstrates that TS levels fall over the fractionated course of RT, to a maximum low at 72 hours before showing signs of recovery.

The changes in TS enzyme activities between fractions were then analysed using the Anova T test. Table 5.5 shows those changes which were significant. This demonstrates that there is a significant drop of TS after consecutive fractions of RT. Some recovery starts to occur after 8Gy but this is not significant.





**Figure 5.9** Comparison of the ratios of TS activity after a fractionated course of radiation normalised to 0Gy controls (HT29)

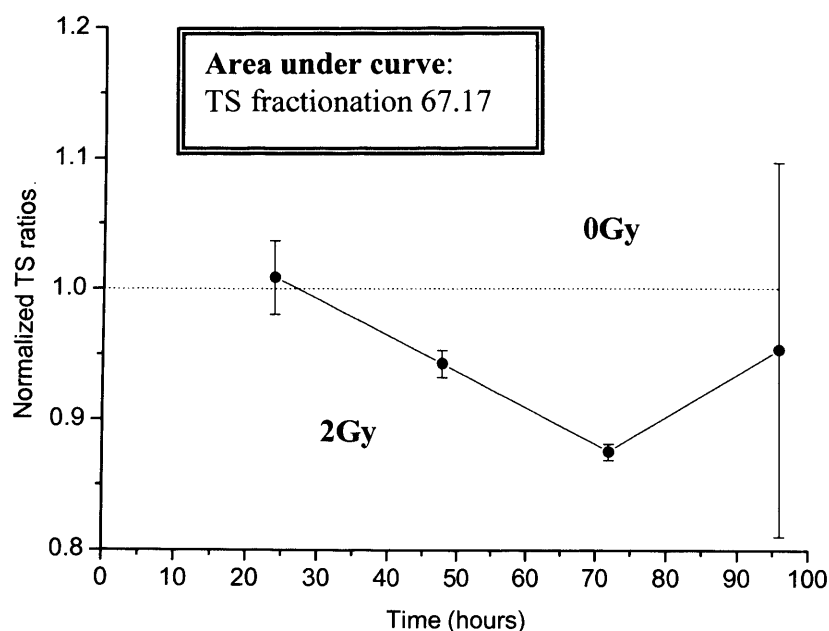
<u>TS activity at Fraction</u>	<u>T test</u>	<u>P value</u>
2 to 6Gy	-12.14	<0.001
4 to 6Gy	-4.08	0.015
2 to 8Gy	-5.00	0.008

**Table 5.5** Significant changes in TS between fractions (HT29)

### 5.4.1.2 SW48

Figure 5.10 shows the ratio of TS activities after 2, 4, 6 and 8Gy normalised to the control levels at each time point for the SW48 cell line. This demonstrates that TS levels fall over the fractionated course of RT, to a maximum low at 72 hours before showing signs of recovery which is exactly the same as in the HT29 cell line. The changes in TS enzyme activities between fractions were analysed, significant changes are shown in Table 5.6. There is clearly a significant drop in TS levels over 6Gy, with some recovery at 8Gy which is not significant.

The results show that TS levels fall during a fractionated course to 6Gy in both cell lines. The levels then show a slight rise at 8Gy, which is not significant. There appears to be no significant difference between the responses in each cell line. This can be seen in the very similar values for area under the curve.



**Figure 5.10** Comparison of the ratios of TS activity after a fractionated course of radiation normalised to 0Gy controls (SW48)

<u>TS activity at Fraction</u>	<u>T test</u>	<u>P value</u>
2 to 4Gy	-2.20	0.090
2 to 6Gy	-4.63	0.010
4 to 6Gy	-5.53	0.005

**Table 5.6** Significant changes in TS between fractions (SW48)

## 5.4.2 Thymidine phosphorylase

### 5.4.2.1 HT29

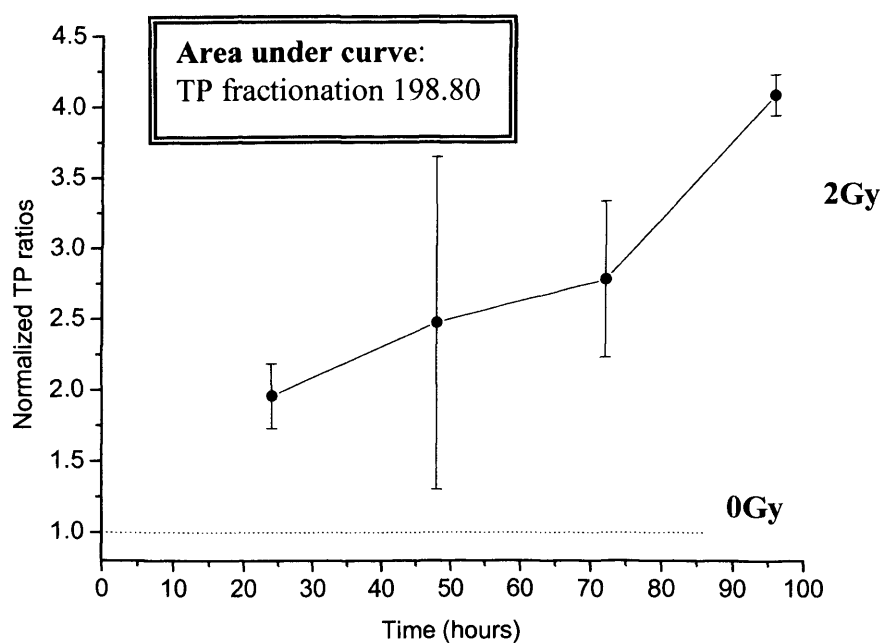
Figure 5.11 shows the ratio of TP activities after 2, 4, 6 and 8Gy (equivalent to 24, 48, 72 and 96 hour time points) normalised to the controls levels at each time point.

This demonstrates that there is a continuous increase in TP levels following each fraction of RT. On analysis of the differences between TP levels at different doses the rise of TP after 8Gy is significantly higher than the level after 2Gy (T test 7.91,  $p=0.001$ ).

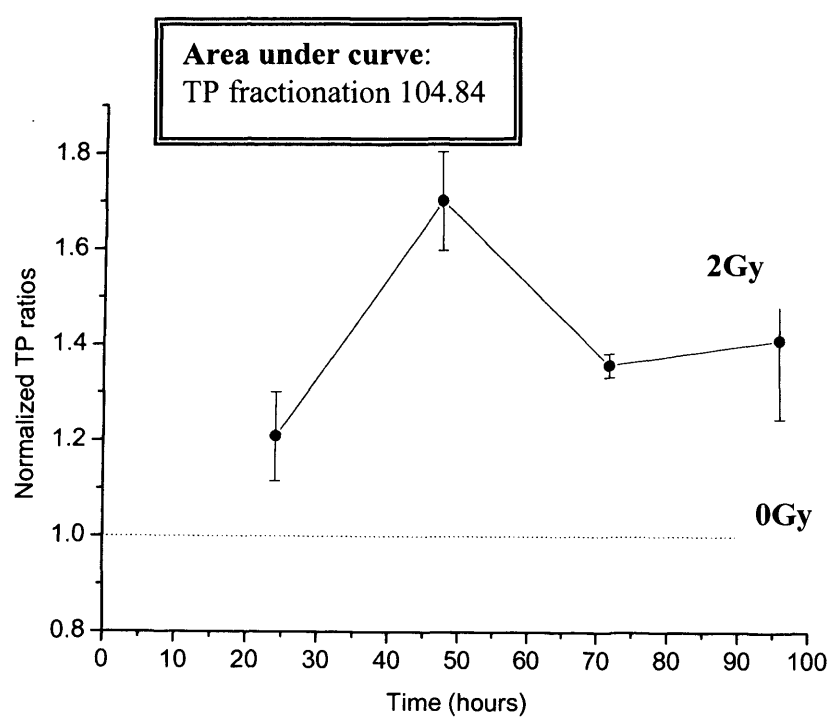
### 5.4.2.2 SW48

In the SW48 cell line there is an increase in TP throughout the fractionated course of RT, see figure 5.12. However, unlike the HT29 cell line where the maximum rise occurs at the end of the RT (8Gy), in the SW48 the most significant rise occurs after the cells have received 4Gy (T test 3.54,  $p=0.024$ ). There is then a significant drop after 6Gy (T test 3.25,  $p=0.031$ ).

In both cell lines however TP clearly increases in response to radiation but it is more prominent in the HT29 cell line; this is demonstrated by the clear difference in area under the curve between each cell line.



**Figure 5.11** Comparison of the ratios of TP activity after a fractionated course of radiation normalised to 0Gy controls (HT29)

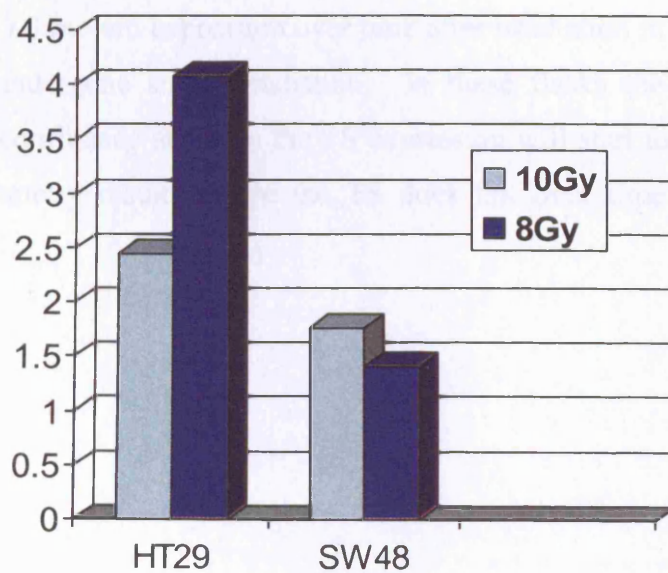
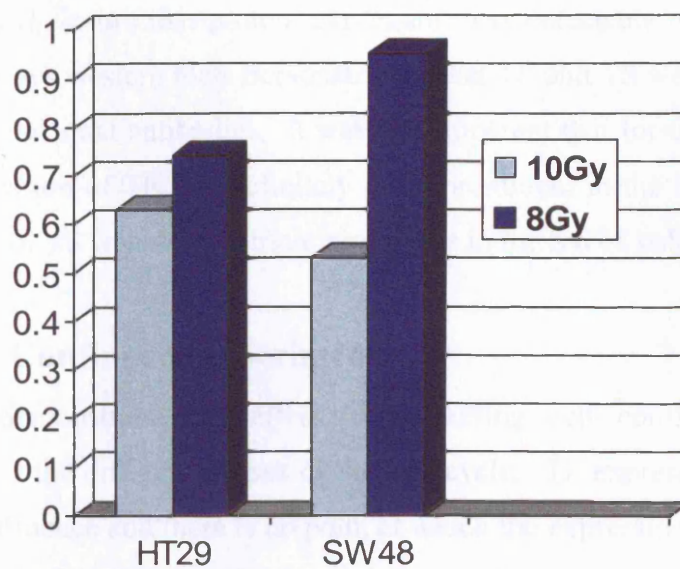


**Figure 5.12** Comparison of the ratios of TP activity after a fractionated course of radiation normalised to 0Gy controls (SW48)

## **5.5 Comparison of enzyme activities between single dose & fractionated RT**

A comparison was made between the ratio of enzyme levels for TS and TP to 0Gy, after a single 10Gy dose at 24 hours and an 8Gy fractionated dose. In the case of TS this revealed that in both cell lines the drop in TS levels at 24 hours after 10Gy was more than after the fractionated 8Gy. This was more prominent however in the SW48 cell line, see Figure 5.13.

TP levels in the HT29 cell line were higher after the fractionated 8Gy, however in the SW48 cells the TP levels were higher after the single 10Gy dose. These differences may be partly explained by the finding that HT29 cells appear to have a higher enzyme level of TP, and SW48 have similar higher enzyme levels of TS.



**Figure 5.13** Comparison of single 10Gy and fractionated 8Gy for Thymidylate Synthase (upper) and Thymidine Phosphorylase (lower)

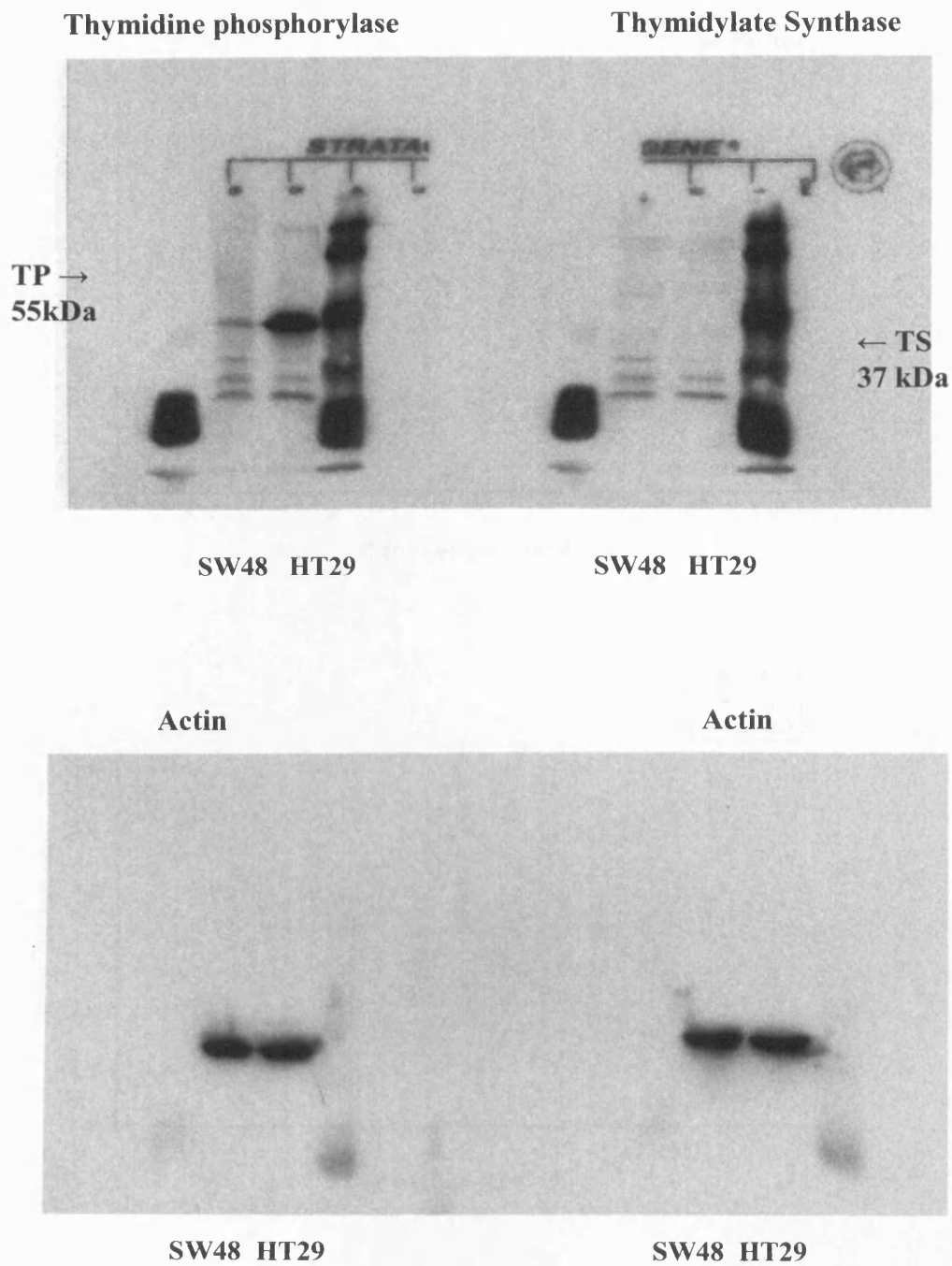
## **5.6 Single dose experiments protein expression**

### **5.6.1 Western analysis**

Prior to commencing the flow cytometry analysis western blots were performed on both cell lines to ensure that protein expression was detectable with each antibody. Figure 5.14 shows western blots demonstrating that TP and TS were detectable using the chosen monoclonal antibodies. It was also apparent that for the same number of cells the expression of TP was definitely more prominent in the HT29 cell line, and the expression of TS was slightly more prominent in the SW48 cell line.

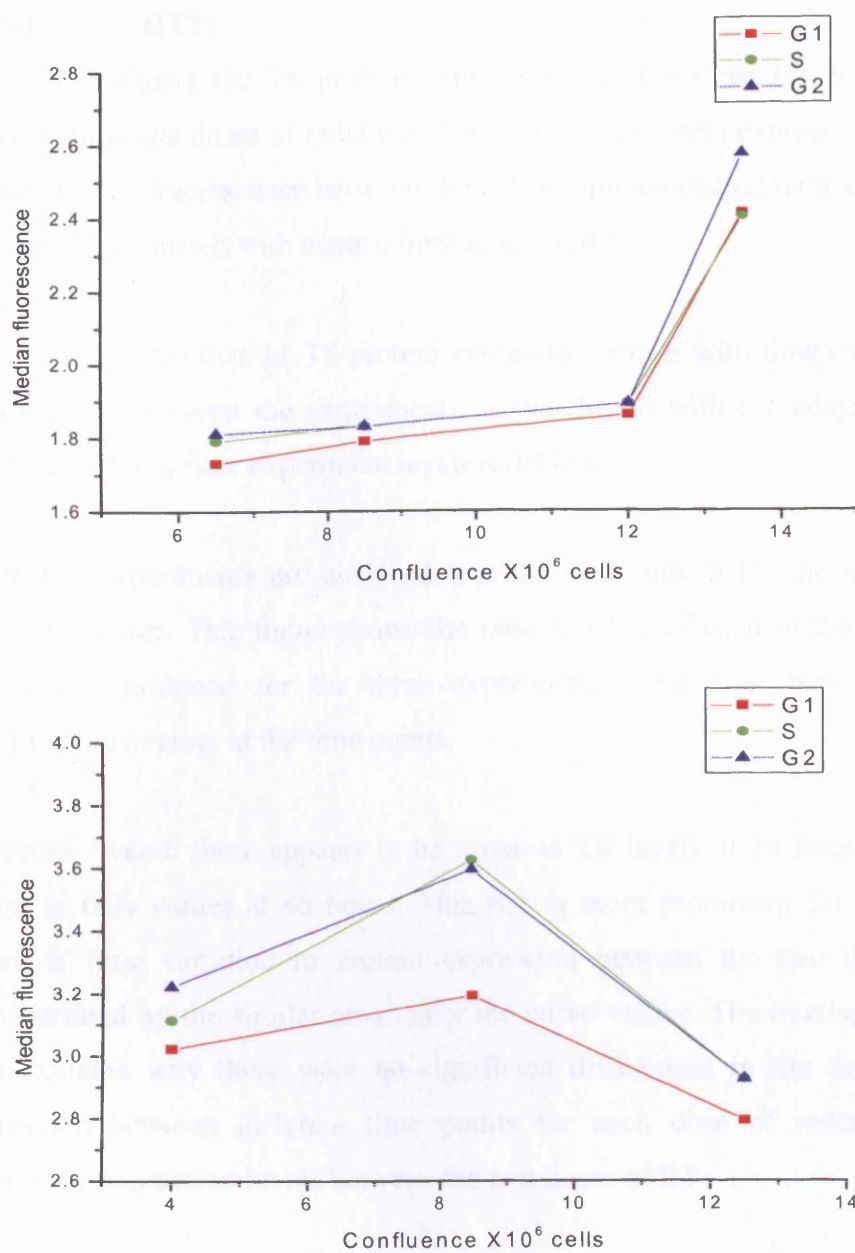
### **5.6.2 Confluence experiments**

Figure 5.15 demonstrates the effect of increasing cell confluence on median fluorescence, in the different phases of the cell cycle. TP expression increases with increasing confluence and there is no point at which the expression levels start to fall, this is true for both cell lines. The increase is seen throughout all phases of the cell cycle. TS expression initially increases with confluence and then falls off dramatically after a confluence of  $\times 10^7$  cells per 75cm<sup>3</sup> flask. This again is true for both cell lines in all phase of the cell cycle. This is particularly relevant when analysing the TS protein expression over time after irradiation in the control samples, which have undergone sham irradiation. In these flasks the cells will continue growing to a confluence at which the TS expression will start to fall. This is seen in the flow cytometry results where the TS does fall over time in the unirradiated controls.



**Figure 5.14** Western blots for TS and TP (upper) and Actin controls showing equal loading of samples (lower)





**Figure 5.15** Effect of confluence on TP (upper) and TS (lower) expression

### **5.6.3 Thymidylate synthase expression**

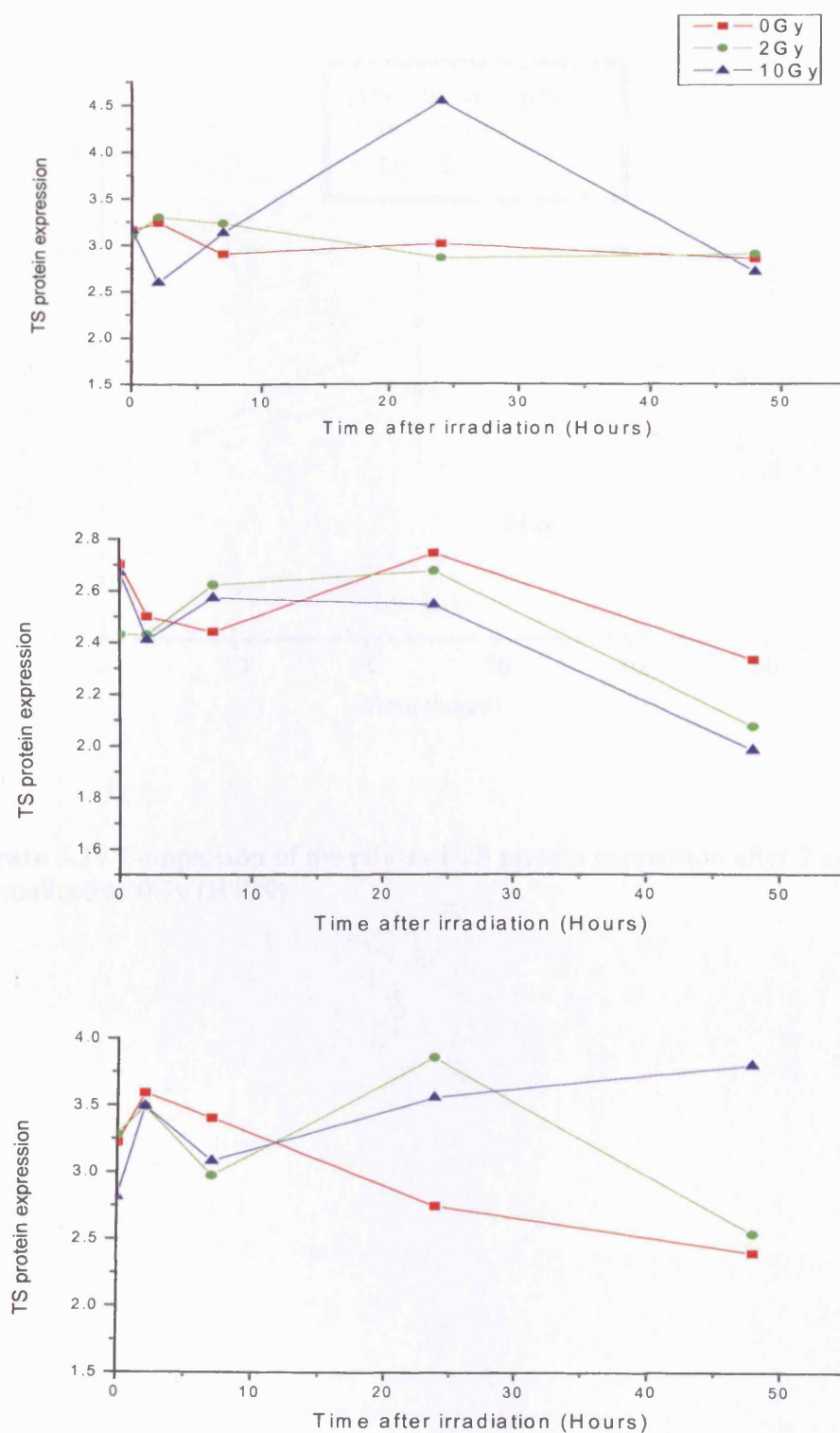
#### **5.6.3.1 HT29**

Figure 5.16 shows the TS protein expressions for the three HT29 experiments in response to single doses of radiation. The amount of protein expression is determined by the ratio of fluorescence between the cell sample incubated with antibody and the cell sample incubated with control immunoglobulin.

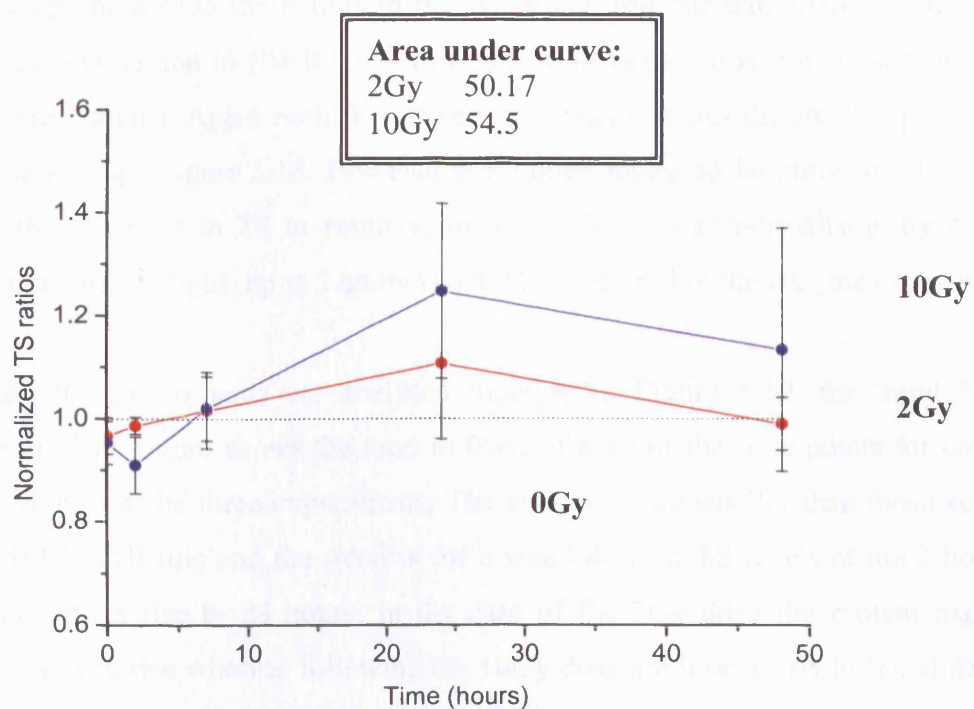
This demonstrates that the TS protein expression results with flow cytometry are not as consistent between the experiments as that found with the enzyme activities in HT29 cells. Each flow experiment result is different.

When the experiments are analysed together in Figure 5.17, the overall response becomes clearer. This figure shows the ratio to 0Gy, of each of the time points for each dose combined for the three experiments. The error bars are large with considerable overlap at the time points.

However, overall there appears to be a rise in TS levels at 24 hours which start to return to 0Gy values at 48 hours. This rise is more prominent for the 10Gy dose. There is little variation in protein expression between the two doses of RT as demonstrated by the similar area under the curve values. The overlapping error bars also explains why there were no significant differences in the degree of protein expression between different time points for each dose of radiation and when comparing expression levels between the two doses of RT.



**Figure 5.16** TS protein expression over time following single dose irradiation in the HT29 cell line



**Figure 5.17** Comparison of the ratios of TS protein expression after 2 and 10Gy normalised to 0Gy (HT29)

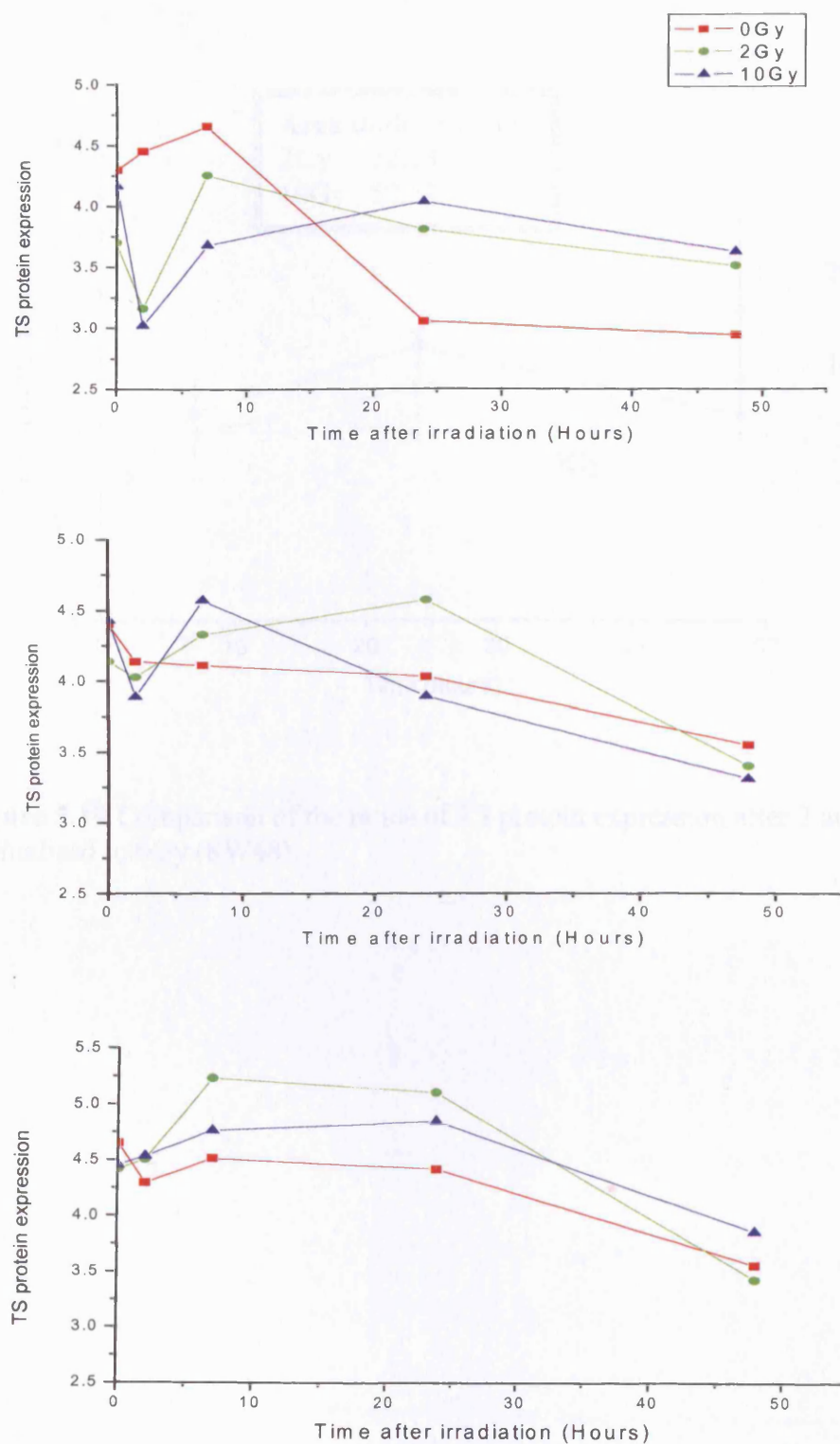
### 5.6.3.2 SW48

In a similar way to the results in the HT29 cell line the data demonstrates that TS protein expression in SW48 cells in response to radiation is not consistent between the experiments. Again each flow experiment demonstrates different responses to the RT doses, see Figure 5.18. However there does appear to be more of a trend in the SW48 with rises in TS in response to RT at 7-24 hours normalising by 48 hours. There is also a slight dip at 2 hours which also occurred in the enzyme experiments.

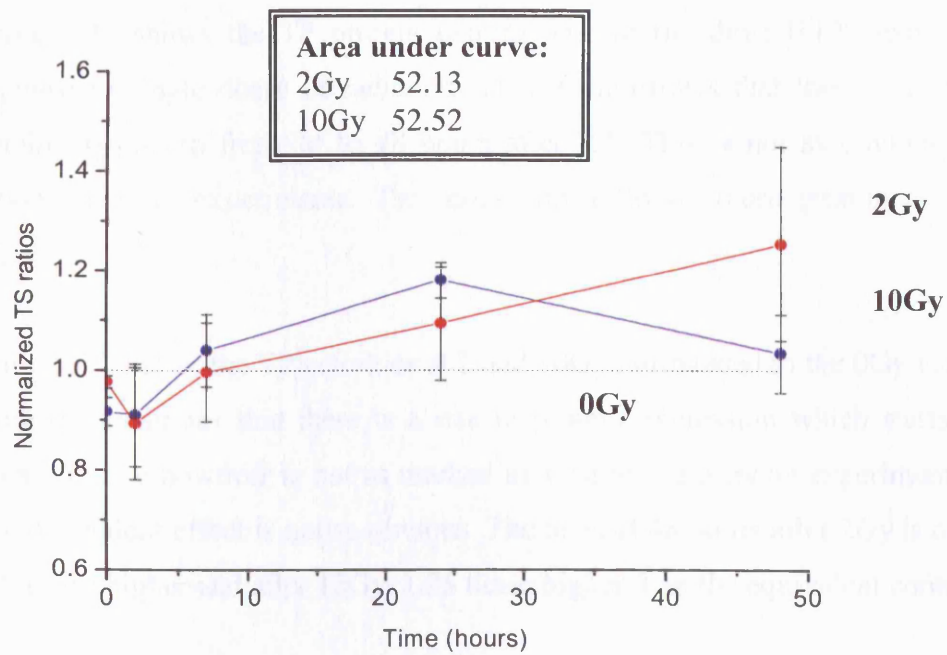
When the experiments are analysed together in Figure 5.19, the trend becomes clearer. This figure shows the ratio to 0Gy, of each of the time points for each dose combined for the three experiments. The error bars are smaller than those seen with the HT29 cell line and the trend is for a small drop in TS levels at the 2 hour time point with a rise to 24 hours. In the case of the 2Gy dose the protein expression continues to rise whereas following the 10Gy dose the level starts to fall at 48 hours. However, no significant differences in protein expression were observed following each RT dose between the different time points.

There is also little variation in protein expression between the two doses of RT as demonstrated by the similar area under the curve values and hence no significant differences were found in the comparisons of TS protein expression at each time point between the two RT doses.

It is clear however that the results with flow cytometry are not as consistent as the enzyme activity assays. The changes in enzyme activity levels are also not mirrored by the changes in protein expression.



**Figure 5.18** TS protein expression over time following single dose irradiation in the SW48 cell line



**Figure 5.19** Comparison of the ratios of TS protein expression after 2 and 10Gy normalised to 0Gy (SW48)



## **5.6.4 Thymidine phosphorylase expression**

### **5.6.4.1 HT29**

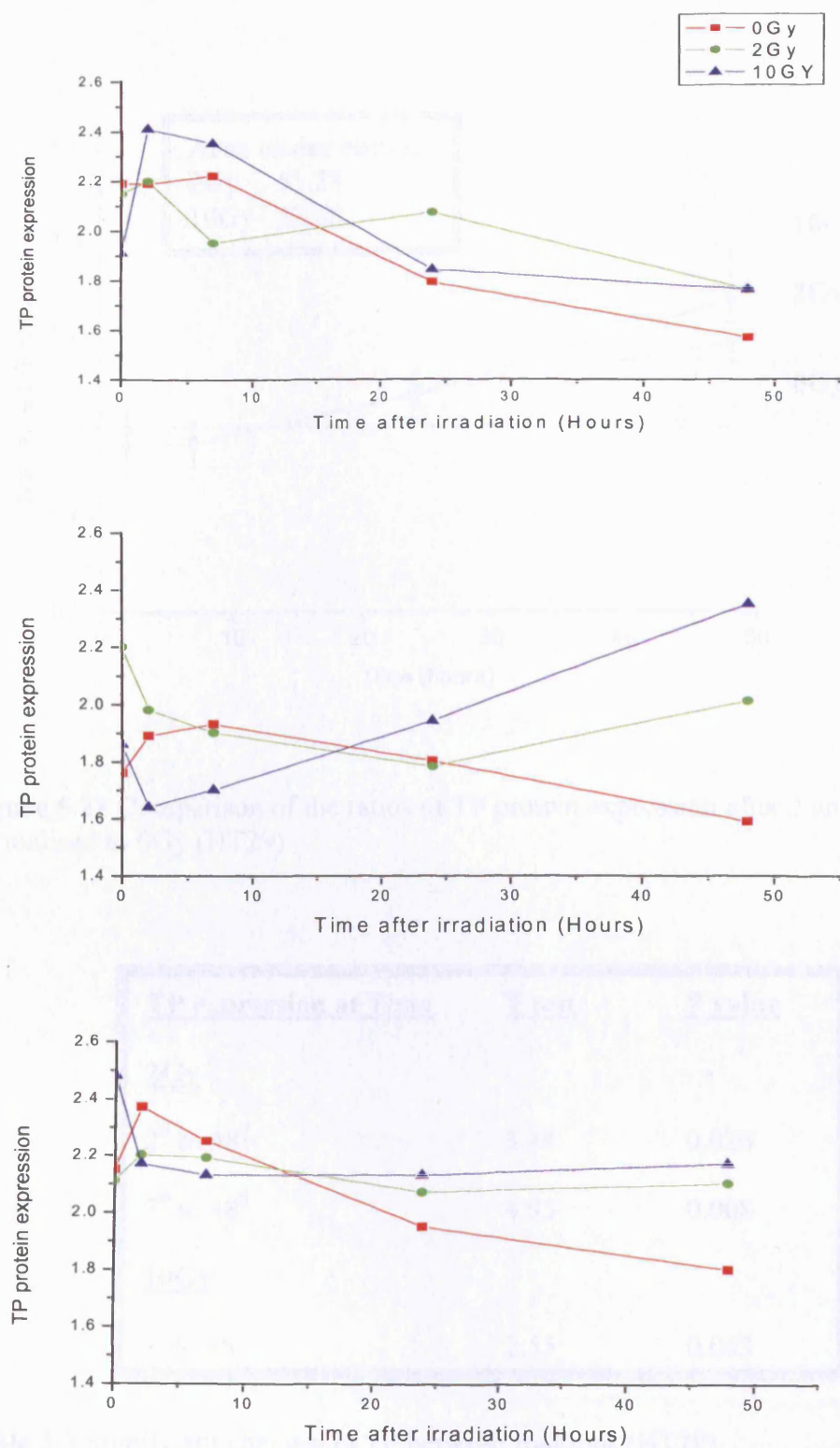
Figure 5.20 shows the TP protein expressions for the three HT29 experiments in response to single doses of radiation. This demonstrates that there is a rise in TP protein expression from 24 to 48 hours after RT. This is not as obvious as in the enzyme activity experiments. This does appear to be more prominent following 10Gy.

Figure 5.21 shows the TP activities at 2 and 10Gy normalised to the 0Gy values. This figure demonstrates that there is a rise in protein expression which starts from 24 hours, the rise however is not as marked as seen in the enzyme experiments and the dose dependent effect is not so obvious. The level at 48 hours after 2Gy is on average 1.18 times higher and after 10Gy, 1.25 times higher than the equivalent control.

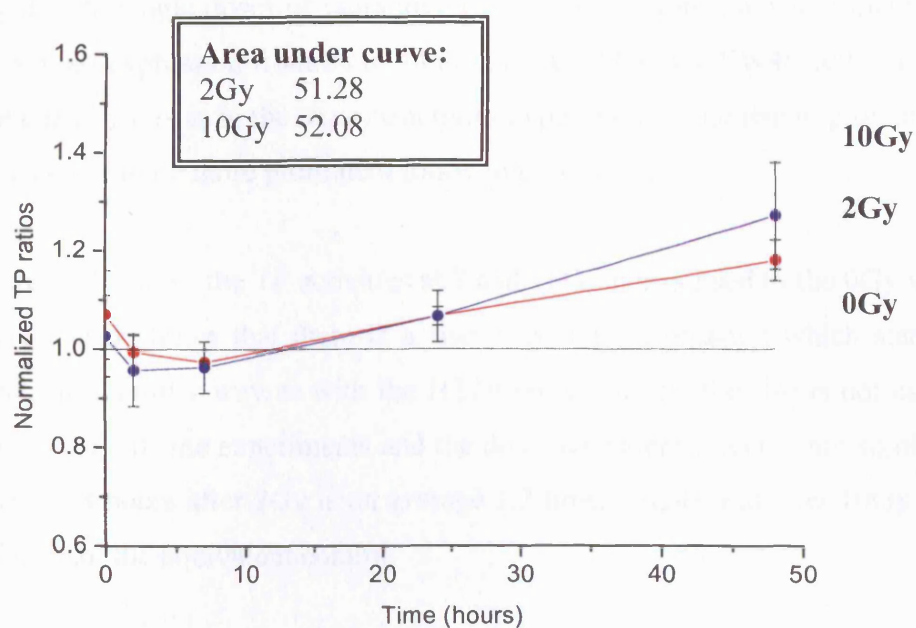
Table 5.7 shows the changes in TP activities between the time points for each radiation dose. The differences between the 2 and 7 hour levels to the 48 hour levels after 2Gy were significantly different. After the 10Gy dose the difference was of borderline significance and this is not surprising because the error bars are larger than those at the 2Gy time points.

A comparison of TP protein expression was also made between the time points for the different doses of RT. There were no significant differences between the two different radiation doses at each time point. This can be seen from the overlapping error bars in Figure 5.21.





**Figure 5.20** TP protein expression over time following single dose irradiation in the HT29 cell line



**Figure 5.21** Comparison of the ratios of TP protein expression after 2 and 10Gy normalised to 0Gy (HT29)

<u>TP expression at Time</u>	<u>T test</u>	<u>P value</u>
<b><u>2Gy</u></b>		
2 <sup>0</sup> to 48 <sup>0</sup>	3.44	0.026
7 <sup>0</sup> to 48 <sup>0</sup>	4.95	0.008
<b><u>10Gy</u></b>		
7 <sup>0</sup> to 48 <sup>0</sup>	2.55	0.063

**Table 5.7** Significant changes in TP between fractions (HT29)

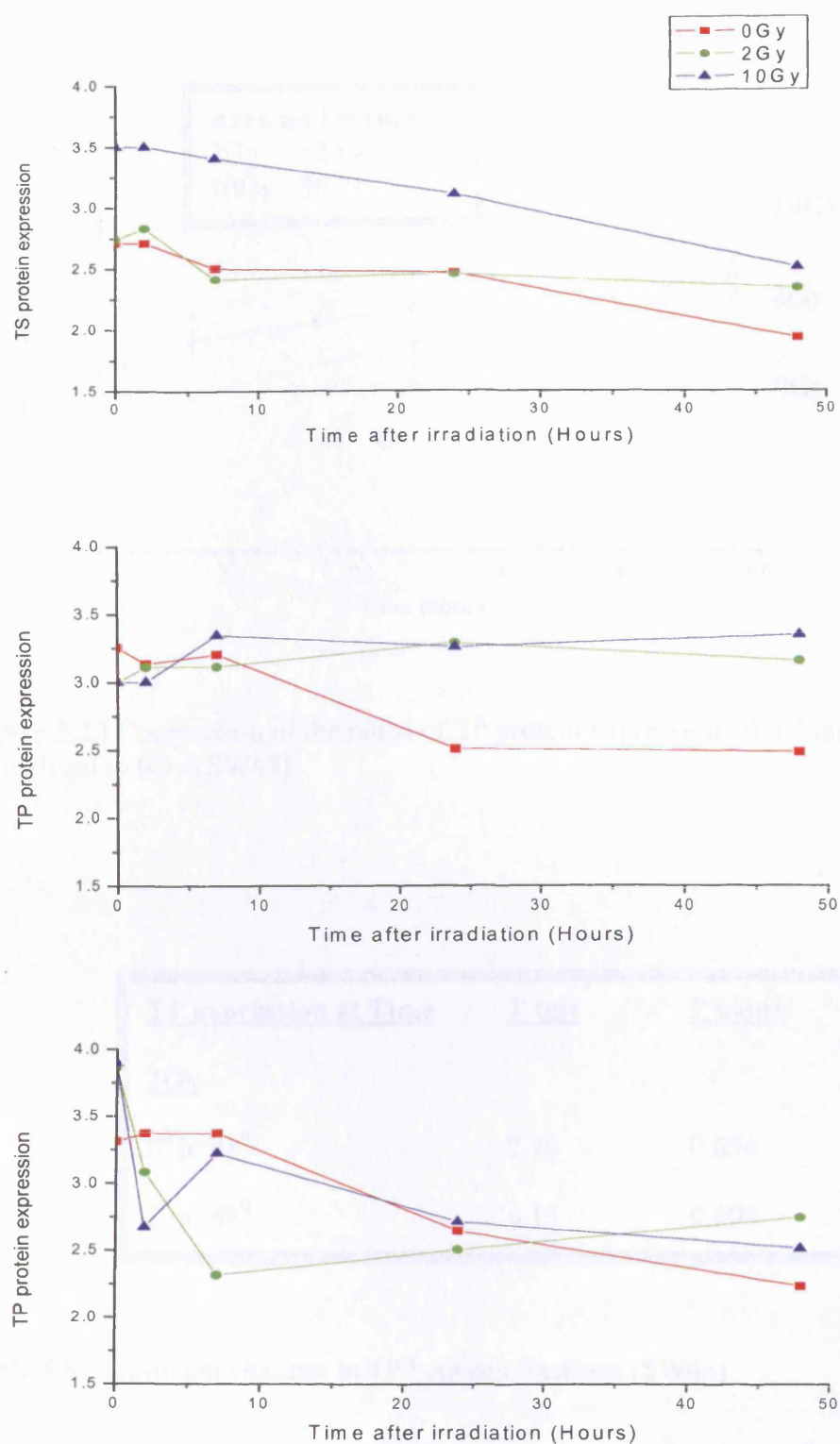
#### 5.6.4.2 SW48

Figure 5.22 shows the TP protein expressions for the three SW48 experiments in response to single doses of radiation. This figure demonstrates that there is a rise in TP protein expression from 24 to 48 hours after RT in the SW48 cell line. Again this is not as obvious as in the enzyme activity experiments. The rise in protein expression does appear to be more prominent following 10Gy.

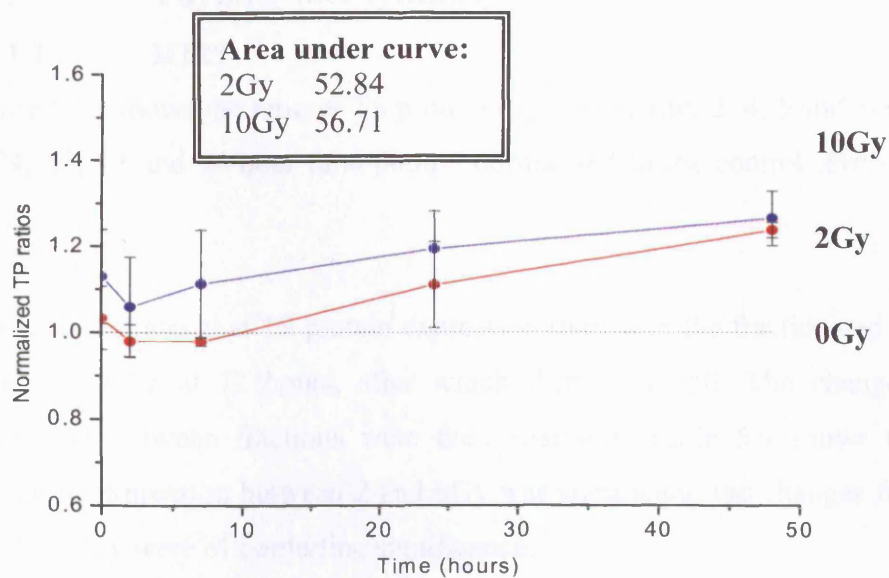
Figure 5.23 shows the TP activities at 2 and 10Gy normalised to the 0Gy values. This figure demonstrates that there is a rise in protein expression which starts from 24 hours. In a similar way as with the HT29 protein levels the rise is not as marked as seen in the enzyme experiments and the dose dependent effect is not so obvious. The level at 48 hours after 2Gy is on average 1.2 times higher and after 10Gy, 1.22 times higher than the equivalent control.

Table 5.8 shows the changes in TP activities between the time points for each radiation dose. The differences between the 0 and 2 hour levels to the 48 hour levels after 2Gy were significantly different. There were no significant differences between the time points following the 10Gy dose. This can be seen from Figure 5.23 where the error bars are clearly overlapping.

A comparison of TP protein expression was also made between the time points for the different doses of RT. There were no significant differences between the two different radiation doses at each time point. Again this can be seen from the overlapping error bars in Figure 5.23.



**Figure 5.22** TP protein expression over time following single dose irradiation in the SW48 cell line



**Figure 5.23** Comparison of the ratios of TP protein expression after 2 and 10Gy normalised to 0Gy (SW48)

<u>TP expression at Time</u>	<u>T test</u>	<u>P value</u>
<u>2Gy</u>		
0 <sup>0</sup> to 48 <sup>0</sup>	2.70	0.054
2 <sup>0</sup> to 48 <sup>0</sup>	6.15	0.004

**Table 5.8** Significant changes in TP between fractions (SW48)

## **5.7 Fractionated experiments protein expression**

### **5.7.1 Thymidylate synthase**

#### **5.7.1.1 HT29**

Figure 5.24 shows the ratio of TS protein expression after 2, 4, 6 and 8Gy (equivalent to 24, 48, 72 and 96 hour time points) normalised to the control levels at each time point.

This demonstrates that TS protein expression rises over the fractionated course of RT to a maximum at 72 hours, after which there is a fall. The changes in protein expression between fractions were then analysed. Table 5.9 shows that only the change in expression between 2 and 6Gy was significant, the changes from 2 to 4Gy and 2 to 8Gy were of borderline significance.

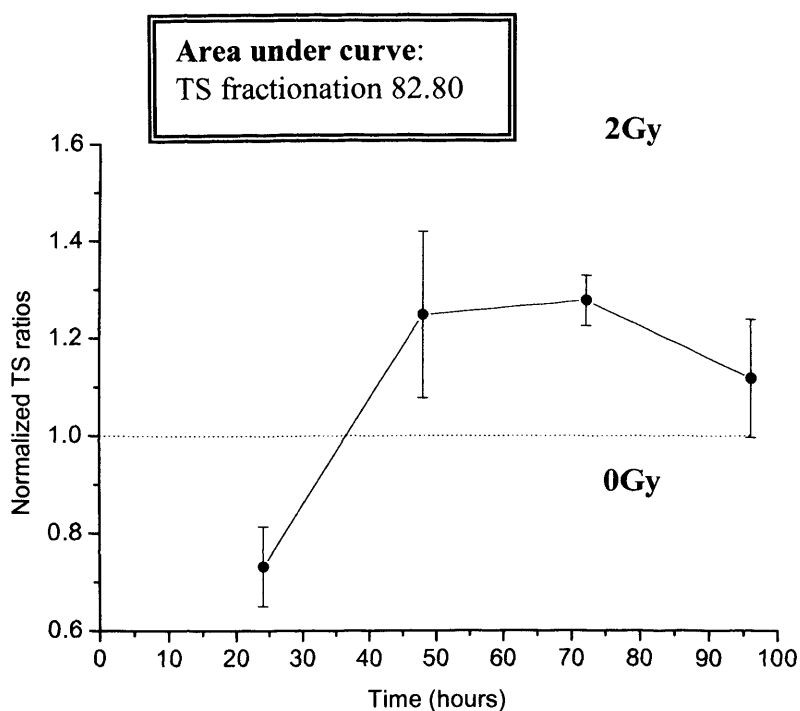
These results are in direct contrast to those seen in the enzyme activity assays where the TS levels fall with fractionation.

#### **5.7.1.2 SW48**

The changes in TS protein expression in the SW48 cell line are similar to those seen in the HT29 cells over a fractionated course. The TS expression increases from 2Gy to a maximum at 24 hours after 6Gy before the levels start to fall. See Figure 5.25.

There were no significant differences in protein expression between the different doses of RT in the SW48 cell line unlike with the HT29 cells.

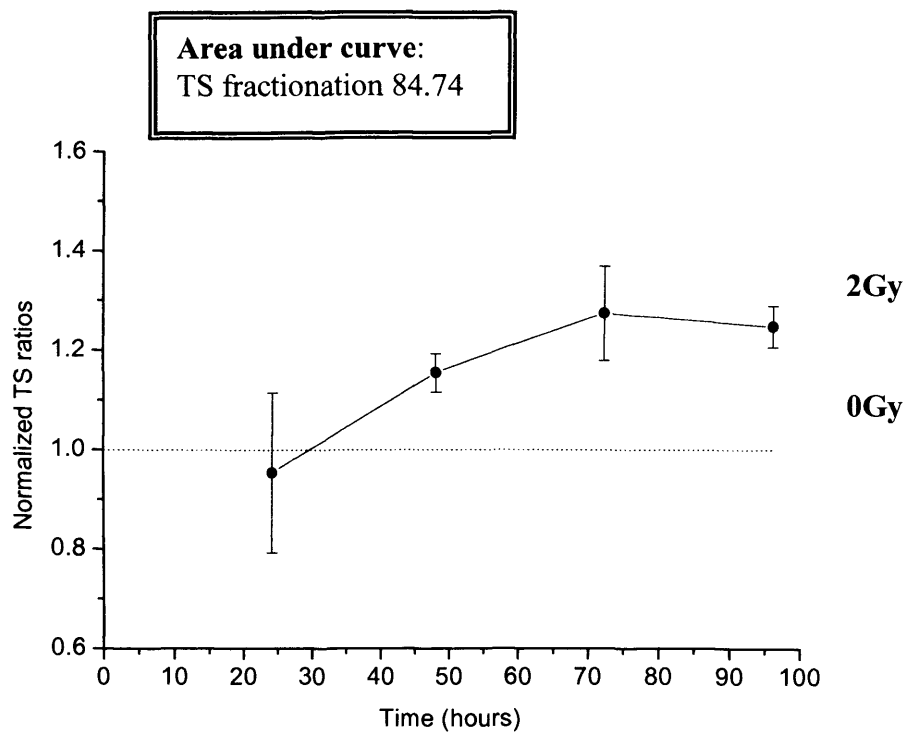
The similar areas under the curve for the two cell lines demonstrate that the changes between fractions are very similar.



**Figure 5.24** Comparison of the ratios of TS activity after a fractionated course of radiation normalised to 0Gy controls (HT29)

<u>TS expression at Fraction</u>	<u>T test</u>	<u>P value</u>
2 to 4Gy	-2.72	0.053
2 to 6Gy	-5.63	0.005
2 to 8Gy	-2.64	0.058

**Table 5.9** Significant changes in TS between fractions (HT29)



**Figure 5.25** Comparison of the ratios of TS activity after a fractionated course of radiation normalised to 0Gy controls (SW48)



## **5.7.2 Thymidine phosphorylase**

### **5.7.2.1 HT29**

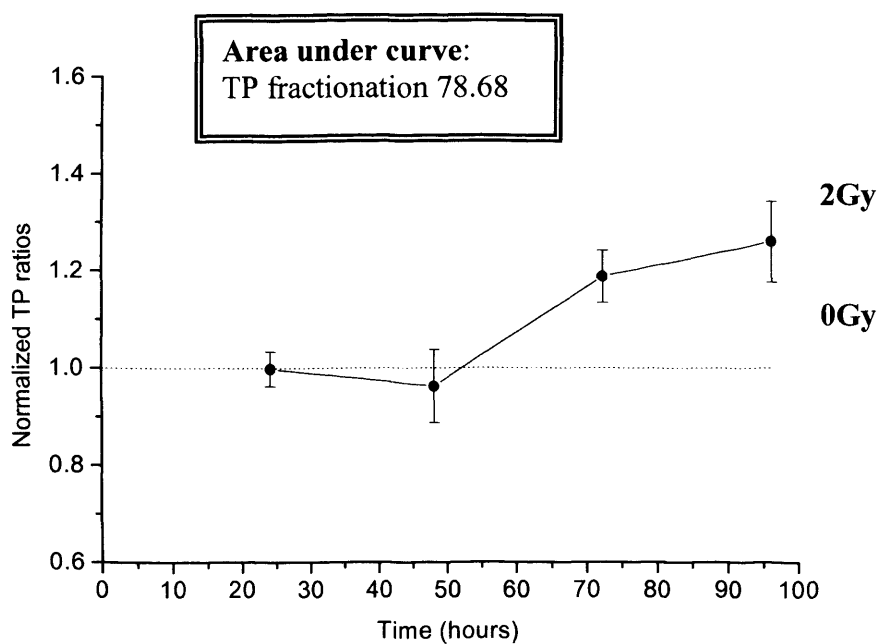
Figure 5.26 shows the ratio of TP protein expression after 2, 4, 6 and 8Gy (equivalent to 24, 48, 72 and 96 hour time points) normalised to the control levels at each time point.

This figure demonstrates that over a fractionated 8Gy course of RT, TP protein expression rises after 4Gy and is still rising 24 hours after 8Gy. These are identical findings to the enzyme activity assays in this cell line but are not as striking. Table 5.10 shows the significant changes in protein expression, which occurred between fractions. In particular the difference between 2Gy with 6 and 8Gy, showing that there is a significant rise in TP over the course of RT.

### **5.7.2.2 SW48**

Figure 5.27 shows the ratio of TP protein expression after 2, 4, 6 and 8Gy (equivalent to 24, 48, 72 and 96 hour time points) normalised to the control levels at each time point.

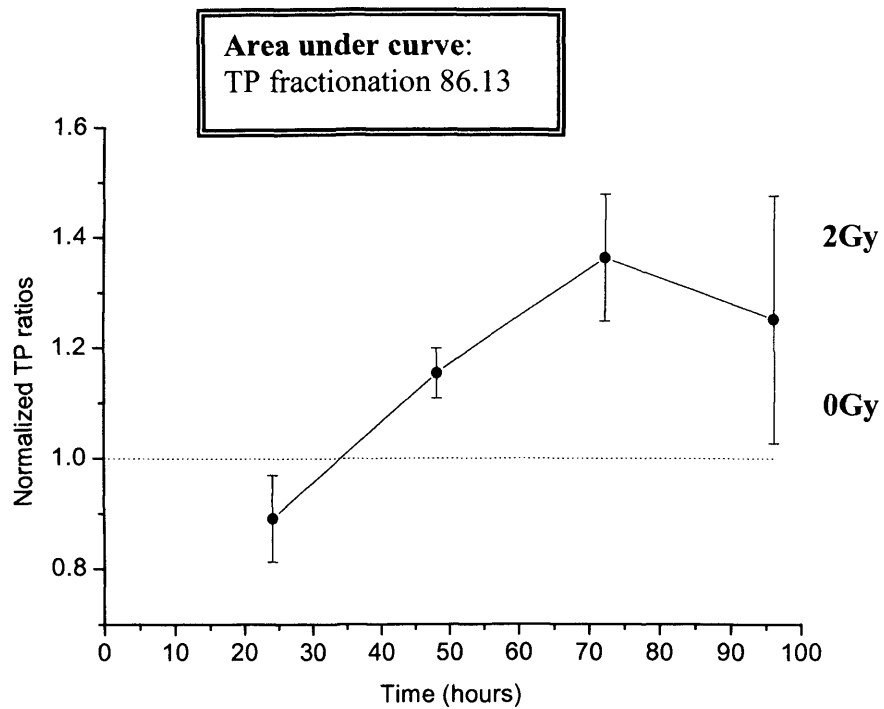
The changes in TP protein expression seen in the SW48 cell line are similar to those in the HT29 cells. The rise in TP occurs earlier after 4Gy rising to a maximum after 6Gy. The TP expression then starts to decrease. These are similar findings to the enzyme activity assays except that the increase in TP expression occurs for a further 24 hours before starting to fall. There were no significant changes in protein expression between the RT fractions. This is demonstrated by the overlapping error bars.



**Figure 5.26** Comparison of the ratios of TP activity after a fractionated course of radiation normalised to 0Gy controls (HT29)

<u>TS expression at Fraction</u>	<u>T test</u>	<u>P value</u>
2 to 4Gy	-2.72	0.053
2 to 6Gy	-5.63	0.005
2 to 8Gy	-2.64	0.058

**Table 5.10** Significant changes in TP between fractions (HT29)



**Figure 5.27** Comparison of the ratios of TP activity after a fractionated course of radiation normalised to 0Gy controls (SW48)

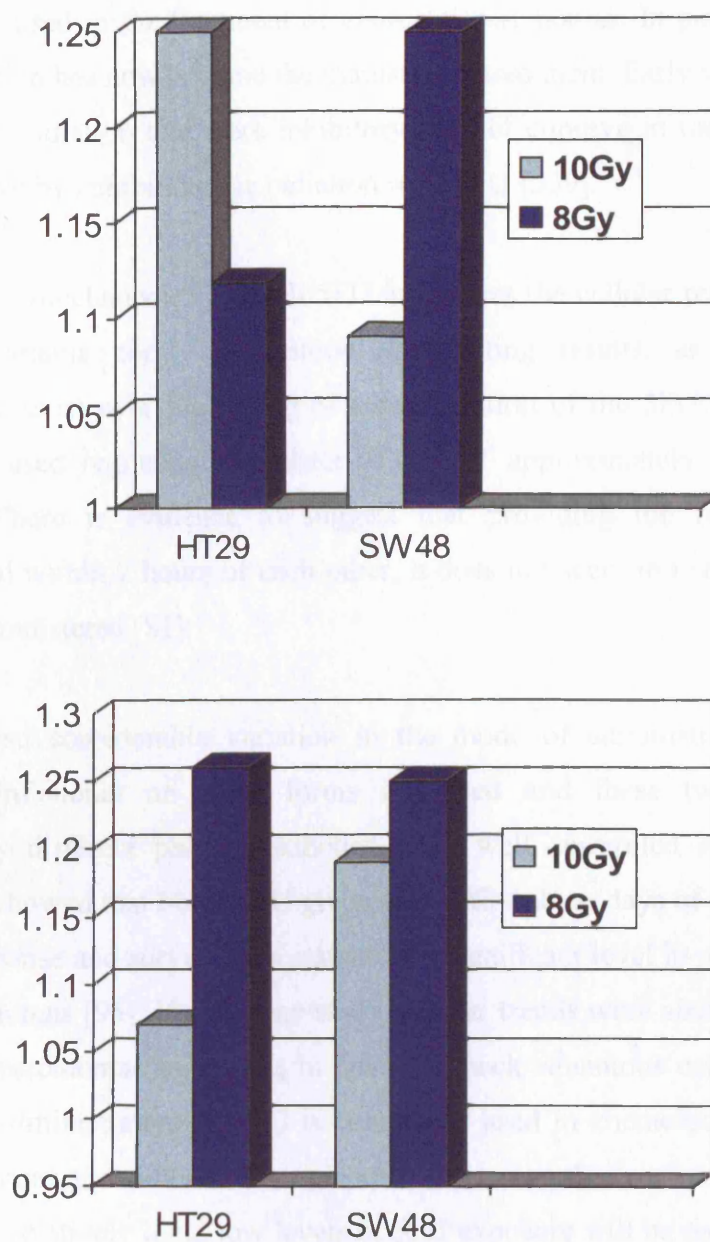
## **5.8 Comparison of protein expression between single dose & fractionated RT**

A comparison was made between the ratio of protein expression for TS and TP to 0Gy, after a single 10Gy dose at 24 hours and an 8Gy fractionated dose at 24 hours, see Figure 5.28.

In the case of TS this revealed that in the SW48 cell line the rise in TS levels at 24 hours after 10Gy was less than after the fractionated 8Gy; however the opposite was true in the HT29 cells.

TP levels in both cell lines were higher after the fractionated 8Gy.

These differences may be partly explained by the finding that HT29 cells appear to have a higher enzyme level and protein expression of TP, and SW48 have similar higher enzyme levels and protein expression of TS.



**Figure 5.28** Comparison of single 10Gy and fractionated 8Gy for Thymidylate Synthase (upper) and Thymidine Phosphorylase (lower)

## 5.9 Discussion

Combined modality treatment regimens of radiation and chemotherapy are being increasingly used in the treatment of anorectal carcinomas. In particular 5FU-based chemoradiation has now become the mainstay of treatment. Early studies showed that the doses of radiation that were inhibitory but not curative in rodent tumours were made curative by combining the radiation with 5FU [339].

However, the mechanism by which 5FU influences the cellular response to radiation however, remains poorly understood. Conflicting results, as discussed in the introduction, exist over the timing of administration of the 5FU with the radiation. Commonly used regimens administer 5FU first, approximately 1-2 hours prior to radiation. There is evidence to suggest that providing the two modalities are administered within 2 hours of each other, it does not seem to matter in which order they are administered [81].

There is also considerable variation in the mode of administration of the 5FU. Currently infusional or bolus forms are used and these two regimens have significantly different pharmacokinetics. In a well controlled study, Moertel and colleagues showed that bolus 5FU given on the first three days of radiation improved tumour response and survival at a statistically significant level in patients with gastric adenocarcinomas [95]. In the same study, similar trends were also seen in pancreatic and bowel carcinomas as well as in head and neck squamous cell carcinomas. This method of administration of 5FU is commonly used in chemoradiation regimens in rectal and upper gastrointestinal carcinomas. However, there is increasing evidence to suggest that relatively long, low levels of 5FU exposure will be required to maximise tumour cell kill in combination with radiation [81]; this has been demonstrated in other *in vitro* studies [340]. Byfield et al [81] suggested that, since the sensitization phenomenon requires at least 24-48 hours to develop, bolus 5FU and radiation are likely to produce only additive effects [81]. In their study the concentration and duration of 5FU required to achieve radiosensitization was similar to the 96-120 hour infusions typically administered in the clinical setting.

In patients who are sensitive to 5FU, the cytotoxic effect mainly depends on the local concentration of the drug that can be achieved within the tumour. However, the systemic administration of 5FU will also result in toxicity to normal tissues, e.g. mucosal epithelial cells of the GI tract or bone marrow cells. One of the strategies for diminishing the side effects of 5FU is some form of selective delivery. Among them, 5'-DFUR is a prodrug which is activated by TP to its cytotoxic form. As has already been discussed TP is elevated in many types of carcinoma cells compared with levels in non-neoplastic regions of the same organ. Potentially this preferential increase in TP in neoplastic cells could be exploited, so that 5'-DFUR is preferentially activated within the tumour cells thus reducing cytotoxic effects on the normal tissues. As a result of this finding the role of suicide gene therapy has been explored and looks promising. Several *in vitro* studies have investigated transfecting neoplastic cell lines with the TP gene, resulting in increased sensitivity to 5'-DFUR [264, 341] [342]. Another strategy to exploit preferential expression of TP in tumour cells is to combine radiation with capecitabine.

Sawada and colleagues [92] demonstrated, using human tumour xenograft models, that TP was up-regulated in response to radiation. Several human cancer cell lines were used including cervical, gastric, colorectal and mammary. Radiation was administered as either a 2.5Gy or 5Gy single dose. Up-regulation of TP was observed in four of the cell lines and it was maximally increased at day nine and then gradually decreased up to day eighteen. The increase in TP was greatest following the 5Gy dose of radiation. In addition increases in TP expression were found to correlate with up-regulation of TNF- $\alpha$ ; TNF- $\alpha$  is a known regulator of TP [343].

Xeloda (capecitabine) is a rationally-designed, orally administered, tumour-activated fluoropyrimidine carbamate. The active drug is formed by a three-step enzymatic conversion to 5FU. In the liver, capecitabine undergoes initial conversion to 5'-deoxy-5-fluorocytidine by carboxylesterase, followed by conversion to 5'-deoxy-5-fluorouridine by cytidine deaminase in the liver and tumour tissue. The final step is conversion to 5FU by thymidine phosphorylase. The interest in capecitabine is the preferential conversion to 5FU at the tumour site exploiting the higher levels of thymidine phosphorylase found in tumour cells compared to normal cells [91]. There may also be a reduction in the radiosensitization and hence toxicity in normal tissues

i.e. an enhanced therapeutic ratio. Sawada demonstrated [92] that when capecitabine or 5'-dFUrd were combined with a single 5Gy irradiation in the WiDr human colon cancer xenograft, the efficacy of the irradiation in combination was much higher than that of either treatment alone. As a result it was concluded that X-ray irradiation would be a rational partner with capecitabine.

There are other distinct advantages to oral Xeloda, in that it is pharmacokinetically similar to infusional 5FU providing a constant level but without the need for a central venous line. It therefore presents a more convenient and patient friendly alternative to infusional 5FU.

The aim of this current study was to confirm that TP is upregulated in response to radiation and establish a dose-time relationship. As well as single doses, a fractionated course was also investigated to mimic the clinical situation. Two colon cancer cell lines were used, HT29 and SW48. The experiments were performed in triplicate and compared to unirradiated controls. The degree of TP activity and protein expression was examined.

In both cell lines TP activity increased significantly following both single doses of radiation and over a fractionated course. The increase in TP activity following the single dose started at 24 hours post irradiation and continued at 48 hours. The rise was more prominent following 10Gy with a level 3-3.75 times higher than in the controls, compared to 1.5 times higher at 2Gy ( $p=0.031$  and  $0.028$ ), showing a dose dependent effect. Following an 8Gy fractionated course there was a continuous increase in TP activity in the HT29 cell line ( $p=0.001$ ). This rise was not as prominent in the SW48 cells and levels started to drop at 6Gy; however they remained significantly higher than the control levels.

These results confirm that TP is upregulated in response to radiation in two different colon cancer cell lines. The upregulation appears to be dose dependent and that a high single dose appears to be equivalent to a similar dose given as a fractionated course. This has not previously been shown.



Protein expression of TP was also examined in this present study using flow cytometry. In a study by Saito [344], also using flow cytometry, TP was expressed by the majority of the colorectal carcinoma cell lines studied but at varying levels. This was mirrored in the present study where the expression of TP was higher in HT29 cells compared to SW48 cells for the same level of confluence.

Comparison of TP protein expression with the enzyme activity revealed similar results but the level of upregulation after irradiation was not as prominent. In both cell lines TP protein expression increased at 24-48 hours following single dose irradiation, and it was more prominent following the higher dose. Following a fractionated course the TP protein expression increased continuously in the HT29 cell line and started to fall after 6Gy in the SW48 cell line. This is similar to the changes observed in the enzyme activity levels. The degree of TP protein upregulation was higher following the fractionated course compared to the single 10Gy dose in both cell lines.

The demonstration that TP is upregulated significantly following a fractionated course of radiation is important, as it is more clinically relevant to current practice and confirms the logical step to investigate the incorporation of capecitabine into current radiation regimens in colorectal cancer.

The other finding in this study was that increasing cell confluence resulted in increased expression of TP in both cell lines. This is consistent with evidence that cytokine release [343, 345-348] or an increasing hypoxic environment or change in the environmental pH [216] can upregulate TP expression. This was not examined as part of this study. In contrast TS protein expression decreased with increasing confluence as the need for DNA synthesis decreased.

In addition to examining the response of TP to radiation the effect on TS was also studied. This was examined due to mounting evidence that the higher TS expression levels correlate with poorer response to 5FU-based chemotherapy both *in vitro* and *in vivo*. In an *in vitro* study of 19 different cell lines, high expression of TS was associated with 5FU resistance [349]. The cell lines most sensitive to 5FU exhibited the lowest TS levels. Similar results have been demonstrated in other *in vitro* studies

[350, 351]. *In vivo* work has demonstrated that high TS gene expression, in patients with colorectal cancer and gastric cancers, was also associated with resistance to 5FU [314]. However, high gene expression levels cannot directly be translated to high TS protein expression [70, 71]. In addition, patients with low intra-tumoral levels of TS on IHC show a higher response rate and longer survival than those with high TS levels after treatment with 5FU for GI cancers [171, 352-354].

The purpose of this study was to examine the effect of radiation on the activity and protein expression of TS. Patients may be less likely to respond to 5FU-based chemoradiation regimens if the TS levels are high or induced by radiation. However, there are few studies investigating the effect of radiation on TS. TS is a key enzyme in DNA synthesis that is activated during S phase and is degraded after the completion of DNA synthesis [355-358].

In this current study, irradiation caused a reduction in TS enzyme activity, in both cell lines, during the first two hours followed by a significant rise at 7 hours, reaching approximately 1.5 times the control level. These changes were not dose dependent. Thereafter, levels fell significantly at 24 hours, more prominently following 10Gy compared to 2Gy, decreasing at 48 hours to 50% of the control activity. However, there was no significant difference between the two doses. Some of this effect may be due to increasing confluence as the TS levels also generally fell over time in the controls. However, the reduction following radiation was significant compared to control levels. This downregulation of TS is likely to be due to decreased proliferation as a result of the radiation. However, this effect with single doses is only likely to last approximately 24 hours, prior to escape from cell cycle delays. This would explain TS activity starting to rise again compared to controls at 48 hours after radiation. In contrast to the present data, Wei and colleagues showed that TS activity increased in response to single doses (0.5, 1.5, 3.75 and 7.5Gy) of radiation in two colon cancer cell lines [359]. This group also found that TS activity decreased temporarily at 3 hours and then rose at 7 hours. However, unlike in this present study where TS activity decreased at 24 hours, they found a dose-dependent increase. The time points were slightly different as were the doses of radiation, however the same activity assay was used. Changes were not more prominent in the HT29 cell line which is more radioresistant than the SW48 cell line, which has been found

previously [359]. A direct comparison of the two cell lines is complicated by their different cell division times which results in different levels of confluency at later times; this affects the unperturbed expression of TS. Also, Western blotting showed that SW48 cells show higher intrinsic levels of TS expression than HT29 cells.

The decrease in TS seen in this study agrees with results from Stammeler et al [360] who showed a decrease in TS mRNA in murine NIH 3T3 cells after exposure to single doses of radiation ranging from 5-20Gy. TS mRNA in this study increased in the first 6 hours post irradiation and then decreased to a minimum at 72 hours.

In the fractionated experiments activity levels of TS significantly fell over the course of the radiation to a maximum low at 72hours. At 96 hours TS levels started to rise, however they were still significantly lower than the corresponding controls. Whether this is an indication of TS levels starting to recover is unclear. These changes were seen for both cell lines and there did not appear to be a dose dependent effect. This downregulation of TS in response to fractionated radiation has been demonstrated previously [361].

When a comparison of the fractionated course was made with the single 10Gy dose the fall in TS activity levels was more prominent following the single 10Gy irradiation. Suggesting there may be some recovery with repeated small dose exposures.

When the protein expression of TS was analysed the changes demonstrated did not necessarily correlate with those changes seen in enzyme activity. The results were not as consistent even between the three experiments. Overall TS protein expression rose at 24 hours following single doses of radiation in both cell lines and then fell at 48 hours. However, in the HT29 cell line there was considerable overlap between the error bars at the different time points and between the two different doses and no significant differences were seen. In the SW48 cells the error bars were smaller and there was a trend for a fall in TS protein expression at 2 hours similar to the enzyme experiments but the subsequent rise continued to 24 hours.

When the fractionated experiments were analysed for TS protein expression the discordance with the enzyme activity changes were more prominent. In direct contrast, the protein expression significantly increased over the radiation course, to a maximum at 72 hours, in both cell lines. There were no significant differences between the two doses demonstrating that the increase did not occur in a dose dependent manner. In the HT29 cell line the increase at 24 hours was more prominent following the single 10Gy dose whereas in the SW48 cell line the increase was more prominent following the fractionated 8Gy at 24 hours.

A major difference between the data for TP and TS was the correlation between enzyme activity and protein expression for the former enzyme and the lack of correlation for the latter. There are few reports correlating protein expression with enzyme activity for either TS or TP. A recent study in colorectal and gastric tumours [273] looked at the relationship between TS measured by enzyme activity, mRNA levels or protein expression assessed by IHC. No linear relationships were found between the mRNA levels and the enzymatic activity and no relationship was found between the protein expression and enzyme levels. This suggests that TS levels vary with the methods used to measure them. These discrepancies must be taken into account when interpreting correlation between TS levels and clinical outcome. However, some studies in cell lines have shown that TS protein content as determined by western blot analysis may correlate with enzyme activity [362]. Also, in a series of colorectal and gastric carcinomas the protein expression of TS on western blot did correlate linearly with gene expression [363]. The factors responsible for these discrepancies remain unclear. Some reports have demonstrated that TS has a complicated transcription mechanism. TS has been shown to interact with its own mRNA to regulate its transcription [364], and polymorphic tandem repeats in the TS gene have been shown to be associated with its protein expression and enzymatic activity in gastrointestinal cancers [365].

These findings may help explain why there are differences in this study between the changes in the enzyme activity and the protein expression of TS. However, unlike in the enzyme activity assays which were consistent and reproducible, the flow cytometric protein analysis was not consistent and may reflect a less reliable method for assessment.

This study has demonstrated that TP is upregulated in response to radiation. This effect is seen not only following single doses but, importantly, following a clinically fractionated course. It is likely that in the future capecitabine will become increasingly used in 5FU-based chemoradiation schedules due the selective delivery and activation in the tumour cells by TP; the activation of TP adds an additional rationale for the combination. Phase I studies have now been completed and phase II studies are underway to investigate the combination of capecitabine with radiation in the treatment of both locally advanced rectal and anal cancer. A recent phase I trial in rectal carcinomas [366] demonstrated that the capecitabine/radiation combination was well tolerated, convenient for patients and easy to administer, simplifying the chemoradiotherapy schedule.

In this study TS activity was shown to decrease in response to radiation which is likely to reflect its role in proliferation. If this is in fact the case its role in promoting resistance to 5FU may not be as important in the context of 5FU-based chemoradiation regimens. This may well explain the lack of prognostic significance for TS in either the anal or rectal series presented earlier in this current study.

# **Chapter 6:**

## **General Discussion**

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## Chapter 6      General Discussion

Optimising locoregional control, disease-free and overall survival remains a challenging goal in the management of adenocarcinoma of the rectum and anal carcinomas. Although significant therapeutic gains from radiotherapy alone and combined chemoradiation have been demonstrated in a number of trials, therapeutic outcome is far from satisfactory.

Advances in our knowledge of tumour biology have led to a rise in studies of new biological markers in many different tumour sites. The aim is to identify markers with prognostic value or predictive of treatment response, so that patients can be selected, on the basis of the biology of their tumour, for more aggressive therapy or for alternative treatment modalities. To date none of these markers have been established in routine clinical practice either in colorectal cancer or anal cancer to aid the prescription of treatment. One reason for the lack of success in establishing a definitive role for these new biomarkers is the poor methodological quality of many studies reported in the literature. For most markers, published studies report diverging or inconclusive results, due to biological, technical and tumour-related differences.

### 6.1 Methodology

There are several available methods for measuring these markers including; biochemical assays, quantitative reverse-transcriptase polymerase chain reaction (RT-PCR), and IHC. The use of biochemical assays is limited to prospective clinical studies requiring fresh or fresh-frozen tissue, and they require a relatively large amount of tissue [367]. In addition quantitative RT-PCR cannot distinguish gene expression in tumour tissues from that in adjacent normal tissues. IHC is thus a favourable method because of its convenience for routine clinical studies and because it can distinguish relatively easily tumour from normal tissues. Ultimately the usefulness of these biological markers in clinical decision-making depends on easy determination at the lowest possible costs. In colorectal cancer, some newly proposed markers such as microsatellite instability [368, 369] appear promising, but not all laboratories are equipped for this analysis.

The selection of the primary antibody and application of an antigen retrieval system to detect the antibody are of great importance in optimising IHC [105]. An example of this is highlighted by the current issue of p53 staining using IHC. Several technical considerations may obscure a relationship between p53 expression and the endpoint of interest. These include variable definitions of a positive p53 IHC result, different antibodies being used with some detecting both normal and mutant epitopes of p53 [370], and different techniques for tissue fixation and antigen retrieval, which may all influence the sensitivity of the IHC assay [371, 372]. Also mutations in the p53 gene may take the form of point mutations, deletions or insertions, resulting in abnormal or no p53 protein being produced. Some p53 mutations result in negative immunostaining, or a wild-type p53 gene may be associated with p53 overexpression [370, 373]. These disparities can also be applied to most of the current markers being analysed in clinical studies and these discrepancies may give rise to some of the disparate prognostic results from IHC studies.

## **6.2 Quality and classification of staining**

One of the main factors contributing towards disparity between studies is the lack of consistent reporting on the quality and classification of staining. There is often considerable variation in the degree of staining, which is considered sufficient for a positive result. Within different regions of a particular tumour, the number and intensity of positive cells appear to vary, which makes it difficult to characterise the actual status of the tumour as a whole, this has been noted by Baas [105] for p53 staining. This has resulted in the case of p53, for studies to use different arbitrary cut-offs for negativity and positivity. Some studies have [114] used 70% or more nuclei staining as positive for p53, whereas others [117] used greater than 10% nuclei as positive staining.

The character of positive staining also varies among the studies. Some quote only one method of scoring the positivity and others use both intensity and percentage. There is no consensus on which is the most important. For this reason in this current series of anorectal carcinomas both the intensity and percentage of cells staining positively were recorded and analysed for each marker. There was no one predetermined percentage cut-off for positivity. For the proliferation markers cyclin A and Ki-67 only the percentage of cells were recorded as this is generally considered



the most important for these markers. For all the other markers; both intensity of staining and percentage of cells staining were scored, and they correlated significantly with each other, with a p-value <0.001 in all cases. It may therefore not be unreasonable to focus on one staining scoring system for each marker, as they do correlate significantly.

However, studying these molecular markers using IHC also highlights other potential difficulties in interpretation. Different tumour types for example have quite significantly different staining patterns. TP staining in the squamous cell anal carcinoma samples clearly demonstrated nuclear, cytoplasmic and stromal expression. In contrast in the rectal adenocarcinoma tumours almost all the positive staining occurred within the stromal cells, this has been demonstrated by others [203]. The importance of these different TP localisations is unclear as described in previous chapters and in fact the predominant role of TP in these two carcinomas may be different. However, it does lead to interpretation difficulties in both the scoring methods and comparisons of the importance of TP between different tumour types. TS also stained differently between the two tumour types with almost all of the anal tumours staining positively but in the rectal tumours the percentage of cells expressing TS varied quite significantly. Scoring systems should be validated for each molecular marker in individual tumour types and comparisons between different tumour sites should be guarded.

### **6.3 Effect of treatment**

Treatment modalities will also affect the clinical importance of some of the molecular markers. In particular some markers have a clear relevance to certain treatments; in particular in this series to preoperative 5FU-based chemoradiation. This is highlighted by the findings of TP in the anal series. In the whole group of patients and the radiation alone arm, increasing TP expression significantly predicted for a poorer DFS. However, in the combined modality treatment arm this effect was not seen and those patients whose tumours strongly expressed TP had an equivalent DFS to those whose tumours expressed no TP. Differences between the two treatment arms can also be seen for the effect of CD34 in this series. It is therefore very important to interpret the importance of certain molecular markers in the context of the treatments patients have received in each study. For example; TP may be a negative prognostic

factor by virtue of its angiogenic properties in patients receiving surgery alone or radiation but in the context of 5FU treatment this may not be the case. Comparisons across all rectal carcinoma series, regardless of the treatment modalities used, may well explain some of the contradictory results seen with certain markers.

A similar point is the issue of using biopsy or surgical specimens to analyse markers with IHC. From the rectal carcinoma series it can be seen that certain markers; in particular the proliferation indices, bcl-2 and EGFR are altered in response to CRT. This may well lead to some markers assuming more importance and others less, by virtue of the possible pre-surgical treatment the patient may have received. More correlations were seen with bcl-2 in the surgical specimens compared to the biopsy specimens. It is important therefore to also take this issue into account when determining the clinical relevance of these markers.

Discrepancies between previous studies may also be due to whether a tumour biopsy or a surgical specimen has been analysed. Analysing biopsies may not be representative of the tumour as a whole. Tumours are very heterogenous and molecular marker expression may differ considerably across the tumour specimen. In particular the expression of CD34 and CA9 may be difficult to assess on a biopsy alone and this may account for the lack of prognostic and response significance in the anal cancer series. CA9 staining for example occurs especially around areas of necrosis and at the periphery of the tumour and this may not be adequately assessed on small tumour punch biopsy specimens. For the anal cancer series however, the surgical specimens would have only been available on those patients who required salvage surgery.

The importance of molecular markers may also change over time because of the introduction of new radiation and chemotherapy schedules. As part of the UKCCR trial, from which the anal carcinoma patients for this study were selected, a six week gap between the two radiotherapy phases was implemented. A gap of six weeks was typically recommended between large-field pelvic and small-volume boost treatment, to allow resolution of acute cutaneous and mucosal toxicity, and to allow assessment of tumour response. This now would be a criticism of the study as groups have identified that this gap may worsen local control [231, 374, 375]. This may well

affect the relevance of some of the findings in this series and it will be interesting to see the future results in ACT II where this treatment gap has been eliminated.

#### **6.4 Statistical analysis**

One of the other major criticisms of studies to date investigating the role of molecular markers is that the statistical analyses are often not robust and most studies are retrospective and small with varying lengths of follow-up. A recent report by Riley et al [96] who performed a meta-analysis of prognostic factors in neuroblastoma highlighted the poor statistical analyses for many of these prognostic marker trials. They identified several problems; no appropriate statistical analysis performed, no hazard ratios reported, inexact p-values reported and marker studies too small. In the current series presented here robust statistical analyses have been used with hazard ratios. In particular in the anal series the numbers are not small and they have been recruited from a randomised trial.

The identification of factors that reflect biological behaviour of individual cancer tissues correlating with tumour aggressiveness is a key determinant of prognosis and a fundamental issue for the improvement of cancer therapy. Despite recent progress in defining the molecular mechanisms of cancer development and tumour progression, only a few individual molecular biomarkers providing prognostic information have been identified.

Tumour response to combined modality treatment is heterogenous and can be influenced by differences in pretreatment size, differentiation, and the biological properties of the individual lesions. If it was possible to identify patients with radio-responsive tumours at the time of diagnosis, a selective and individualised policy of preoperative chemoradiation could be pursued. This would be highly clinically relevant. In rectal carcinomas it might be possible then to consider less radical surgery with possible sphincter preservation in low lying tumours or even a local resection without lymphadenectomy in very early stage lesions.

Despite all the current potential problems molecular markers are likely to play a role in the future in the treatment decision-making process in patients with anorectal carcinomas. The most promising from this current series are p53, CD34, TP and

EGFR. Already trials are underway looking at the role of capecitabine in both anal and rectal chemoradiation regimens and this is as a direct result of its activation by TP. A recent phase II study of cetuximab, an EGFR monoclonal antibody, in metastatic colorectal cancer has shown good response rates and improved progression-free times.

However issues of inconsistent assay methods and patient and treatment heterogeneity, which detract from the ability to draw definitive conclusions, must be addressed. Ideally future studies using larger cohorts of uniformly staged and treated patients to define the clinical usefulness of these promising markers should be undertaken. Wherever possible the analysis of these markers from patients entered into phase III randomised clinical trials should be performed. Finally for these potential prognostic factors to be clinically useful there must be practical and reliable assays that can be broadly available to all centres with agreed validated IHC protocols for each marker.

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